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STUDIES ON THE EPIDEMIOLOGY AND CHEMICAL CONTROL OF FUSARIUM SEEDLING BLIGHT OF WHEAT USING MOLECULAR TECHNIQUES

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A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

2002

Harper Adams University College in collaboration with Syngenta

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ABSTRACT

Fusarium seedling blight is an important disease of wheat caused primarily by seed-borne *Microdochium nivale* of which there are two sub-species; var. *nivale* and var. *majus*. Though important pathogens of UK wheat crops, little is known about the relationship between seed-borne inoculum of each sub-species and subsequent disease, annual and regional distributions of inoculum, their pathogenicity as seed-borne pathogens or the relative effectiveness of the main method used to control seedling blight (seed treatments). This investigation aims to answer questions relating to these important factors of *M. nivale* as a seed-borne pathogen. Nucleotide sequences for the elongation factor 1-α gene were used to produce primers specific to *Microdochium nivale* var. *nivale* and var. *majus*. Internal standards were constructed and quantitative PCR assays developed using standard assay curves. *Microdochium nivale* var. *nivale* and var. *majus* inoculum was quantified on seed harvested in 1997, 1998 and 1999. Total *M. nivale* DNA was significantly related to the determination of *M. nivale* contamination using standard agar plate counts in each year, both estimates of *M. nivale* contamination were significantly related to seedling blight disease symptoms. A positive correlation was observed between fungal biomass of *M. nivale* var. *nivale* and var. *majus* in each year. *Microdochium nivale* var. *majus* was the dominant sub-species present in Scottish samples from each year though only in English samples from 1999. *Microdochium nivale* var. *majus* was the more pathogenic seedling blight pathogen as either inoculated surface-borne or natural seed-borne inoculum. Beret Gold was most active of six fungicide seed treatments towards isolates of each sub-species *in vitro*. Field trials showed that the amount of *M. nivale* DNA quantified in seed was reflected in the amount present in the resultant seedlings. This was reduced significantly through the use of the seed treatments Beret Gold or Sibutol. Significant differences between the treatments were evident when disease pressure was high. *Microdochium nivale* var. *majus* was the most effective coloniser at the seedling stage whereas var. *nivale*

predominated prior to flowering. These results show that (i) PCR assays are a useful determinant of *M. nivale* contamination of wheat seed and seedling blight symptoms, (ii) seasonal and regional fluctuations occur in sub-species predominance on seed samples (iii) *M. nivale* var. *majus* is a more pathogenic seedling blight pathogen than var. *nivale* (iv) Sibutol and Beret Gold are effective against both sub-species at the seedling stages of crop development.

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Edwards, S.G., Hetherington, R., **Glynn, N.C.**, Hare, M.C., West, S. J. E. and Parry, D. W. Evaluation of fungicide seed treatments against Fusarium diseases of wheat using PCR diagnostics tests. *Brighton Crop Protection Conference 1998 – Pests & Diseases* 1017-1022.

Glynn, N. C., Edwards, S. G., Hare, M. C., Burke, R. and Parry, D. W. Quantification of Fusarium spp. and M. nivale in fungicides seed treatment efficacy trails6th European Fusarium Seminar 2000, Berlin p40 (Abstract of oral presentation).

Glynn, N. C., Edwards, S. G., Hare, M. C., Burke, R. and Parry, D. W. Assessing the field performance of seed treatments for the control of Fusarium seedling blight of wheat using quantitative PCR B*righton Crop Protection Conference 2000 – Pests & Diseases 479-482.*

Glynn, N. C., Edwards, S. G., Hare, M. C., Parry, D. W. and Brandl, F. Studies on the incidence and control of Fusarium seedling blight of wheat caused by *M. nivale* var. *majus*, var. *nivale* and *Fusarium* spp. using PCR diagnostics. *British Crop Protection Council Conference 2001 - Seed Treatments – Challenges and Opportunities 231-234.*

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION AND LITERATURE REVIEW

TAXONOMY

The genus *Fusarium* contains several plant pathogens that occur on cereals in the UK. The most important of these are *F. culmorum, F. graminearum, F. avenaceum* and *F. poae.* Until 1983, the genus *Fusarium* also included *F. nivale.* Samuels and Hallett (1983) investigated the taxonomy of the genera *Gerlachia, Monographella, Micronetriella* and *Fusarium.* As a result of their work, *F. nivale* was re-classified into the genus *Microdochium* due to its holoblastic-annellidic conidiogenesis. Previously, Arx (1981) had described conidiogenesis within the *Microdochium* genus as being holoblastic-sympodial. Both types of proliferation are found in a variety of other fungi (Samuels and Hallett, 1983) and as no differing conidia ontogeny is observed as a result, the type of proliferation was therefore not considered important with respect to taxonomy. Work by Wollenweber (1931) identified two sub-species within *F. nivale. Fusarium nivale* var. *majus* was described as having wider conidia than var. *nivale.* Gerlach and Nirenberg (1982) described var. *nivale* as being in the main 1-3 septate whilst var. *majus* isolates could be distinguished as they had predominately 3 septate conidia. Interestingly, these workers recorded no discriminating differences in other morphological characteristics. In agreement with this, Nelson *et al.* (1983) found no differences in spore morphology between isolates within *M. nivale.* Similarly, Litschko and Burpee (1987) found no distinct biotypes within *M. nivale* on the basis of conidial morphology, conidiogenesis, fungicide response (*in vitro*), growth rates at 10 and 20°C or asexual compatibility among thalli. In contrast, Niessen (unpublished) found evidence suggesting stronger pathogenicity to wheat in var. *majus* than var. *nivale* (Gams, 1989).

In a review of *Fusarium* taxonomy Windels (1991) concluded that molecular techniques would aid in the classification of *Fusarium* and related species by confirming species boundaries which were at the time based on morphological characteristics. Lees *et al.* (1995) supported Windels' (1991) assertions. Using Random Amplified Polymorphic

DNA (RAPD) analysis they showed considerable homogeny between UK isolates of *M. nivale* which fell mainly into a corresponding group based on conidial width. This group concurred with those descriptions by Wollenweber *et al.* (1931) and Gerlach and Nirenberg (1982) of *M. nivale* var. *majus* that produced wider, predominantly 3 septate conidia. Lees *et al.* (1995) also identified a second group that correlated in the main to var. *nivale.* Greater variation was noted between isolates assigned to var. *nivale.* Similar observations were made by Maurin *et al.* (1995) on the basis of esterase isozymes and polymorphisms within the Internal Transcriber Spacer (ITS) region of ribosomal DNA (rDNA) in continental European isolates. These corresponded to UK isolates identified by Lees *et al.* (1995) as *M. nivale* var. *majus* defined using RAPD analysis. A large degree of heterogeneity was recorded in the remaining isolates. To distinguish them from the uniform sub-group var. *majus* and in the absence of further information, they were named var. *nivale.*

Lees *et al.* (1995), unlike Maurin *et al.* (1995) were able to distinguish most isolates assigned to relevant sub-species on the basis of conidial morphology. In the study by Lees *et al.* (1995), one isolate assigned var. *nivale* on the basis of conidial width showed an RAPD profile identical to that shown by var. *majus.* On the basis of conidial morphology therefore, the boundaries which existed between the two sub-species appeared to overlap, however, using RAPD analysis, boundaries between the two sub-species become more defined. Improved taxonomic divisions can, therefore, be achieved through the use of analytical and molecular technologies alongside conventional techniques.

The high degree of variation noted within the two *M. nivale* sub-species, particularly var. *nivale* has been discussed by several workers (Parry *et al.*, 1995b; Lees *et al.*,1995; Maurin *et al.*, 1995). In these studies, perithecia were produced by isolates designated to both var. *majus* and var. *nivale* and it was suggested that both sub-species share homothallic reproduction. The high occurrence of polymorphisms within isolates

assigned var. *nivale* was described as being due to the ability of that group to also reproduce heterothallically in nature (Lees *et al.*, 1995).

FUSARIUM DISEASES OF WHEAT

Fusarium spp. have been isolated from a variety of plant and soil materials throughout the world including Africa (Tarr, 1962), Asia (Roy, 1974; Kelman and Cook, 1977), Australia (Burgess *et al.,* 1987), Europe (Tusa *et al.,* 1981; Baker, 1972) and North America (Gordon, 1959; Ingilis and Cook, 1981). It appears from the literature therefore that *Microdochium nivale* is almost exclusively associated with graminaceous plants.

In the UK, fourteen *Fusarium* spp. have been isolated from wheat crops. Of these only *F. culmorum, F. avenaceum* and *F. graminearum* are regarded as virulent pathogens. *Fusarium culmorum*, together with *M. nivale* were found in a survey by Paveley *et al.* (1996) to be by far the most common. *Fusarium poae,* though rarely directly the cause of serious crop losses is a mycotoxin producer and was noted by Polley and Turner (1995) as being the predominant cause of glume spot lesions on wheat in the UK in 1989-1990. *Fusarium* spp. and *M. nivale* commonly cause three diseases of cereals; Fusarium seedling blight, Fusarium foot rot (or brown foot rot) and Fusarium ear blight (scab).

Fusarium Seedling Blight

Fusarium seedling blight is recognised through a range of symptoms. These vary from pre- and post-emergence death of seedlings (Figure 1.1a) to browning of the coleoptile and the development of superficial stem lesions on the emerged plants (Figure 1.1b) (Jenkins *et al.,* 1988) resulting in reduced plant stands in infected crops. According to Parry *et al.* (1994) the first leaf often takes on a shredded appearance following infections by *Fusarium* spp. or may be seen lying on the surface of the soil and in some cases lens-shaped lesions appear on both the first and second leaves. Infections caused by *M. nivale* often become visible following snow thaw and is known as 'snow mould' where seedlings

are usually deformed into a corkscrew shape if they manage to penetrate the soil surface (Anon., 1998).

Fusarium Foot Rot

Early symptoms of *Fusarium* foot rot in winter wheat are usually present during spring in the form of decay of the lowest leaf sheath. This may become shredded, blackened and fall onto the soil surface (Parry *et al.,* 1994). As the stem degrades, dark perithecia often appear in the brown areas of the remaining lower leaf sheaths. Infection of the stem-base is usually confined to the lower 3 nodes and becomes more apparent as the crop approaches anthesis. The infection often girdles the lower internode and appears as brown or black streaking of the higher internodes (Figure 1.2) (Parry *et al.*, 1994). Severe infections may result in lodging of the crop as the stem-base becomes rotten and breaks. The characteristic, pink mycelium of the fungus may be visible if the stem is split open (Anon., 1998). According to Parry (1990) and Polley and Turner (1995), the most common cause of *Fusarium* foot rot in the UK is *M. nivale* although *F. avenaceum* and *F. culmorum* have been isolated from stem-base material in the UK. The latter, along with *F. graminearum*, are more frequently the cause of foot rot infections in warmer climates such as Australia (Burgess *et al.,* 1975) and the US (Cook, 1968).

(a)

(b)

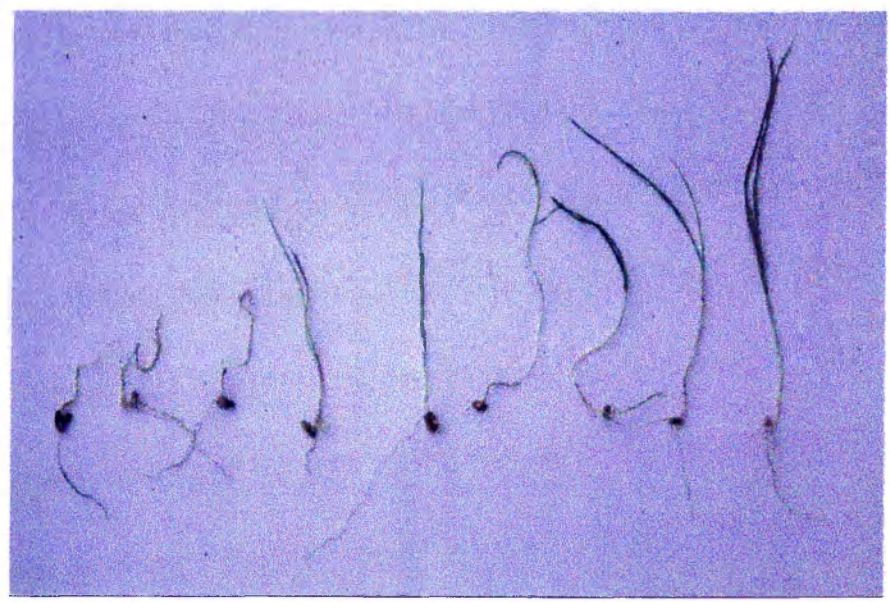


Figure 1.1 Effects of Fusarium seedling blight on (a) seedling emergence (fungicide treated seed plot left, untreated seed of same seed lot right) and (b) Fusarium seedling blight symptoms on individual seedlings (severely infected left, healthy seedling right).

Figure 1.2. Symptoms of Fusarium foot rot on winter wheat.

***Fusarium* Ear Blight**

Fusarium ear blight is first evident when pale brown water-soaked lesions appear on the outer glumes. These spread to the point of glume attachment to the rachis. As the infection matures, the ear takes on the premature colouration of a ripe head, possibly due to restricted water and nutrient transport (Figure 1.3). At an advanced stage of infection, white mycelium, together with pink/orange sporodochia is often seen (Parry *et al.,* 1994). Although a range of *Fusarium* spp. are implicated in causing the disease, three species predominate in temperate regions: *F. graminearum*, *F. culmorum* and *F. avenaceum* (Parry *et al.,* 1995a). Rapilly *et al.* (1973) noted brown glume spots with dark brown margins caused by *M. nivale*. In contrast, Cassini (1981) stated that ear infection caused by *M. nivale* could only be detected by reduction in thousand grain weight. Thus, ear infections caused by *M. nivale* would appear to be symptomless.

The three *Fusarium* diseases that occur on small grain cereals in temperate climates are represented in Figure 1.4. This generalised disease cycle proposes that each disease can provide a source of inoculum for subsequent infections.

Figure 1.3. Symptoms of Fusarium ear blight on winter wheat.

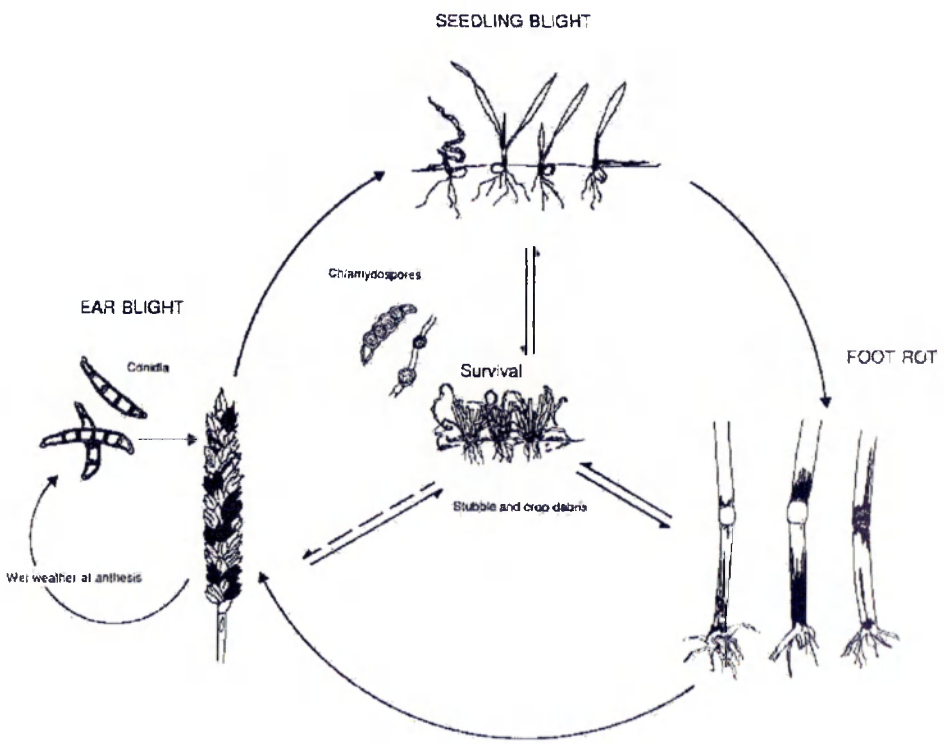


Figure 1.4. Generalised *Fusarium* disease cycle on small grain cereals (from Parry *et al.* 1994).

SOURCES OF INOCULUM FOR SEEDLING DISEASES

Seed-borne inoculum

Fungal contamination of cereal seed can be due to the presence of spores on the seed surface or mycelium infection within the seed. Oxley and Jones (1944) confirmed the earlier findings of Bolley (1913) that the mycelium of a variety of fungal species including *F. culmorum* present within healthy, mature grains of wheat exists under the epidermis. In an investigation into the position and entry of fungal inoculum present in wheat seed, Hyde and Galleymore (1951) found *Fusarium* spp. among the most common species present on the outer beard region though they did not state which species of *Fusarium* spp. they identified. They also concluded that fungal inoculum present within the seed was more abundant at the beard end than the basal embryo region of the seed. Though extracellular in the main, Hyde and Galleymore (1951) found some intracellular penetration of the epidermal cells though again the authors failed to speciate the *Fusarium* they found. Srobar and Srobarova (1978) observed the inoculum of *F. culmorum* to spread both inter and intracellularly in grains of winter wheat. They stated that depending on the depth of colonisation, fungal inoculum may be present in the epicarp, endocarp and embryo.

Colhoun (1983) stated that although the total spore load on grain was important, further spores are located away from the embryonal zone, the less the risk of seedling infection. Elekes (1983) observed *Fusarium* spp. in the pericarp, endosperm and testa of wheat seed, however, the aleurone layer and embryo remained free from infection. Again the author failed to state which *Fusarium* species were observed. Bateman (1983) was able to isolate *M. nivale* from the outer epidermis, inner pericarp and testa and, to a lesser extent from the embryo and endosperm of naturally infected wheat seed. Cristani (1992) stated, however, that the most virulent and abundant infection caused by *M. nivale* was located in the outer seed layers. The findings of Hyde and Galleymore (1951), Srobar and Srobarova (1978), Elekes (1983) and Colhoun (1983) appear to indicate that the amount of fungal inoculum present on infected grain is important. Also, the position of the inoculum

on seed appears to be a contributing factor when considering the subsequent infection of seedlings.

Soil –borne inoculum

Initiating the *Fusarium* seedling blight and foot rot depends upon the successful survival of inoculum. This can be seed or soil-borne, however, the survival structures have been shown to differ between species.

Snyder and Nash (1968) found *F. culmorum* to be the predominant species present in soils where cereals were grown in the U.K. suggesting that this was due to the abundance of chlamydospores produced by this species. *Fusarium culmorum* was not isolated from soils where root crops were grown or from uncultivated woodland. *Fusarium avenaceum* was isolated more frequently from what they termed 'the uncultivated wilderness' than from soils where wheat was grown. Snyder and Nash were able to isolate *M. nivale* frequently from the above ground cereal plant parts, however they were unable to isolate the fungus from any soils. This, they suggested, was due to the inability of *M. nivale* to produce chlamydospores.

In agreement with the work of Snyder and Nash (1968), Rawlinson and Colhoun (1969), were unable to isolate *M. nivale* directly from soil using selective media containing pentachloronitrobenzene. However, the pathogen was readily isolated from seedlings grown in the same soil from seed that had previously been shown to be free from *M. nivale.* Both Snyder and Nash (1968) and Rawlinson and Colhoun (1969) concluded that *M. nivale* survives from year to year as macroconidia and vegetative masses on infected wheat straw, humus or on the soil surface. Snyder and Nash (1968) showed that on infected wheat straw which was dried slowly, *M. nivale* could survive in a paper envelope in the laboratory for as long as 3 years.

Booth and Taylor (1976a) planted *M. nivale* inoculated wheat seed 1, 2, 3 and 4 weeks prior to sowing healthy, uninfected wheat seed at 25mm distances from the

inoculated seed. Obvious disease symptoms developed only in those seedlings where the mycelium had already extended through the soil into which they were planted and not on those seedlings sown in soil through which the mycelium grew after seed germination. They noted that the growth of *M. nivale* was far quicker from seed inoculum than from straw. In a further investigation, the same workers observed that *M. nivale* produced no spores on either seed or straw within the soil, however, *F. culmorum* produced macroconidia profusely. The failure of *M. nivale* to produce conidia in soil they suggested indicated the absence of any survival role for these spores.

Alternative hosts as a source of inoculum

The association of *Fusarium* spp. and *M. nivale* with cereal crops has been well studied over many years, however the association of the same fungi with other plant species is less well represented in the literature.

Jenkinson and Parry (1994) isolated five *Fusarium* species (*F. culmorum, F. graminearum, F. avenaceum, F. poae* and *F. sambucinum*) from the stem-bases of 1346 broad-leaved weed plants comprising 15 different weed species from agricultural fields in the UK. They observed no Fusarium disease symptoms on the host plants, however, 75 out of 77 of the isolates obtained were pathogenic towards wheat seedlings. The authors suggested that broad-leaved weeds could provide an important source of *Fusarium* spp. inoculum for infection of wheat crops particularly in the case of *F. poae* which they stated is rarely isolated from either crop debris or wheat culms. Jenkinson and Parry (1994) were unable to isolate *M. nivale* from any of the species of weed investigated. Khan and Ahmad (1998) reported that the pathogen was isolated from 0.5 – 11 % of 66 vegetable seed lots from Northern Pakistan analysed using a standard blotter technique. The findings of Kahn and Ahmed (1998) represent the only reported identification of *M. nivale* from anything other than graminaceous plant material or soil. It therefore appears that *M. nivale* infections are almost exclusive to graminaceous plants. *Microdochium nivale* infections of

a variety of cereals and turfgrass have been well reported (Jamalainen, 1959, Vaartnou and Elliott, 1969, Litschko and Burpee, 1987 and Diamond *et al.,* 1995). It is apparent that graminaceous plants may provide an important alternative source of inoculum for the infection of cereal crops.

INFECTION AND COLONISATION

Seedling infection and stembase colonisation

The infection and colonisation of wheat plants by *Fusarium* spp. and *M. nivale* has been observed over many years, from a variety of inoculum sources using a wide range of techniques. Bennett (1928) noted that penetration of developing wheat seedlings by *F. culmorum* and *F. avenaceum* from both soil and seed-borne inoculum occurred through the primary root and shoot. Simmonds (1928) observed root infection of oats by *F. culmorum* to take place below appressoria through root hairs whereas the developing shoot was invaded via the mesocotyl and coleoptile. Once inside the plant, the fungus developed between the coleoptile and plumule. Bennett (1928) recorded that infections moved up the primary stem to the 'crown' from which point it extended into the lowest aerial internode with hyphae being both inter and intracellular. Hyphae were noted by the same author as being aggregated below the epidermis, specifically in cavities below the stomata from which point the hyphae protruded to the exterior through stomata or between epidermal cells. Bennett also observed blocking of the xylem caused by the fungal mycelium being massed in these elements. Bennett stated that this caused restriction of supplies of water from the soil leading to the characteristic 'premature head ripening' symptom. In a further investigation into the colonisation of wheat seedlings from soil-borne *M. nivale,* Bennett (1933) found that the inoculum was present in the moribund and dead plant parts only such as seminal roots and coleoptiles. Malalasekera *et al.* (1973) investigated penetration and invasion of wheat seedlings by *F. culmorum* and *M. nivale.* They reported penetration by *F. culmorum* from inoculated seed occurred through the hypocotyl, coleoptile or both.

They found that the fungus advanced through the primary, first and second seminal roots but was restricted to the basal parts. Rarely did the fungus develop further than 1.5 cm from the hypocotyl. Penetration of the primary root and coleoptile by *M. nivale* occurred after these structures broke through the coleorhiza and pericarp, respectively. Six days after sowing, the authors observed hyphae in the cortical cells of the primary and lateral roots adjacent to the coleorhiza. However, as with *F. culmorum*, infections showed little movement downwards within the root system. Malalasekera *et al.* (1973) also observed intercellular hyphae in the epidermal layer between the coleorhiza and coleoptile. They suggested this, along with penetration by hyphae outside the cuticle of the seed, formed the primary methods by which penetration from seed-borne inoculum occurred. Malalasekera *et al.* (1973) also monitored the invasion of wheat seedlings following inoculation of the coleoptile with spores of *M. nivale*. They noted that conidial germ-tubes spread randomly over the surface of the coleoptile before entering through stomates or breaks in the cuticle. Hyphae branched in the substomatal chamber with further hyphae spreading in all directions which developed into a prosenchymatous mat. Invasion of cells by hyphae was observed via hyphal pegs passing through a cell wall pit though this was rare. The authors observed penetration of the coleoptile cuticle though they could not establish the precise mechanism of penetration. Weste (1975) noted that *F. culmorum* and *F. graminearum* produced larger amounts of the cell wall degrading enzymes pectinmethylesterase and cellulase than other saprophytic colonisers of cereals. They considered these two species of *Fusarium* to be among the most pathogenic towards cereal tissue. Snijders (1990) observed *Fusarium* infections of winter wheat caused by soil-borne *F. graminearum* and *F. culmorum* as high up the stem as the peduncle. The same authors concluded that the solid diaphragm between the internodes was not a barrier to *F. culmorum,* and that from wound infections, the fungus can grow both acropetaly and basipetaly. Diamond and Cooke (1997) used scanning electron microscopy to show that *M. nivale* enters the leaves of several cereal hosts via stomatal colonisation and penetration.

Infection of wheat ears

The infection of wheat ears by *Fusarium* spp. and *M. nivale* as with any plant pathogen is dependant upon the interaction of several factors including the susceptibility of the host, virulence of the pathogen and environmental conditions. Atanasoff (1924) showed that the first ear blight symptoms of the growing season occurred during late anthesis which he concluded was the stage most susceptible to infection. Atanasoff (1924) suggested that spores could remain on the developing ear until heads reached a susceptible stage for infection. In an investigation of ear infection of wheat by *F. avenaceum*, *F. culmorum*, and *F. graminearum,* Dickson *et al.* (1921) showed that the highest incidence of ear infection of wheat occurred at flowering. They found that, infection took place through extruded anthers and then spread to adjacent anthers. De Tempe (1964) concluded that the composition of the *Fusarium* pathogen complex present on infected wheat seed was strongly dependent on the environmental conditions during grain ripening. This concurred with the earlier findings of Hyde and Galleymore (1951) who observed that as the moisture content within developing grain fell during ripening, a corresponding fall in the incidence of sub-epidermal fungi occurred. De Tempe (1964) found that in Dutch wheat seed obtained during what he described as a more or less normal summer (1961), the incidence of *M. nivale*, *F. graminearum*, *F. avenaceum*, and *F. culmorum* was more or less equal. After what he described as a cooler summer in 1962, De Tempe observed over 70% of seed samples to be infected with *M. nivale.*

In an extensive survey of cereal contamination by *M. nivale* and *Fusarium* spp. in Holland between 1962 and 1986, Daamen *et al.* (1991) found the incidence of *M. nivale* on contaminated seed to be correlated with the average temperature in July and August. The contamination of harvested seed was also positively correlated with the average cumulative precipitation between June and August. They suggested that wet conditions during June, July and August may stimulate late ear infection and delay harvest allowing prolonged

infection of seeds. No correlation was observed between contamination of seed sown and infected seed at harvest. Likewise, Humphreys *et al.* (1995) reported that seed-borne *M. nivale* had no direct effect on the degree of infection of harvested grain. Hyde and Galleymore (1951) isolated *Fusarium* spp. from sections of the stem immediately below the ear. Based on this they suggested that systemic infection by *Fusarium* spp. was a cause of contamination of grain. Hutcheon and Jordan (1992) observed ear blight symptoms caused by *F. avenaceum F. culmorum, F. graminearum* and *M. nivale* in wheat plants inoculated at the stembase at growth stage 37 (Zadoks *et al.*, 1974). They suggested that systemic infection had taken place though they failed to demonstrate how it had occurred. At growth stage 37, the developing wheat ear is present within the plant at or near the stem-base (Zadoks *et al.*, 1974), a likely alternative explanation for the findings of Hutcheon and Jordan (1992) would be that inoculation of the stem-base at growth stage 37 resulted in infection of the developing ear which remained with the plant until ear emergence. In contrast, Clement and Parry (1998) in an investigation into fungal colonisation by *F. culmorum, F. graminearum* and *M. nivale* from inoculated compost were unable to isolate any pathogens from the fifth node or ear up to growth stage 95. Using scanning electron microscopy they showed that systemic growth occurred only in senescent plants at harvest and that fungal growth within the culm tissues or pith cavity only extended for what they termed a short distance above discolouration of the stem.

ENVIRONMENTAL FACTORS AFFECTING DISEASE

Much of the work on the environmental factors that affect *Fusarium* seedling and stem based disease-causing pathogens has concentrated on the effects of soil moisture and temperature. Dickson (1923) investigated the influence of soil moisture and soil temperature on the development of seedling blight in spring and winter wheat caused by *F. graminearum*. Little or no seedling blight was observed at soil temperatures below 8°C in glasshouse and field trial experiments. Seedling blight was most severe at soil

temperatures of 16° to 24°C in both spring and winter wheat. The percentage of blighted seedlings was seen to increase with low soil moisture at all temperatures. Bennett (1933) investigated the affect of soil pH on the pathogenicity of *M. nivale*. Soils with pH values of 4.5, 7.1 and 8.9 were inoculated with mycelium of *M. nivale*. Oat, barley and wheat seedlings grown in the soil showed disease symptoms in all soils though these were slightly more severe in acidic soils. Bennett concluded that *M. nivale* could survive and attack developing seedlings equally well in both alkaline and acidic soils. Millar and Colhoun (1969) in an investigation into factors affecting seedling blight of wheat caused by *M. nivale* concluded that disease incidence decreased slightly with increase in soil pH over the range pH 4.8-8.1. Papendick and Cook (1974) showed that in dense stands of winter wheat receiving high nitrogen applications (up to 224 $kgNha^{-1}$) stem-base disease severity was increased. This they concluded was due to depletion of soil water caused by healthy plants with extensive root systems and high transpiration rates caused by increased leaf area index. Soil moisture appears to be important when considering stem-base and emergence diseases caused by *Fusarium* species. Colhoun and Park (1964) investigated the affects of soil temperature (11.9, 17.6 and 22.6°C) and moisture (30, 52 and 71% maximum water holding capacity (M.W.H.C)) on foot rot and pre- and post-emergence seedling blight caused by *F. culmorum*, *F. graminearum* and *F. avenaceum*. Pre- and post emergence death caused by *F. graminearum* and *F. culmorum* was most severe in the driest soils at the two higher temperatures whereas stem lesions were most frequent in dry soils at 17.6°C. The incidence of all diseases caused by *F. avenaceum* was far less than for the other two *Fusarium* spp. under all conditions. In an investigation involving soil temperature, moisture and spore load, Colhoun *et al.* (1968) concluded that for *F. avenaceum* and *F. culmorum* spore load could act as a substitute for unfavourable environmental factors. They observed that a higher concentration of spores was required for *F. avenaceum* than *F. culmorum* to produce the same symptoms. The same authors observed no increase in disease severity caused by *M. nivale* compared to the uninoculated

control under all environmental conditions. Millar and Colhoun (1969) questioned this observation attributing the earlier lack of seedling infection to antagonism by soil micro-organisms. They showed that five out of twenty naturally occurring soil micro-organisms were antagonistic towards *M. nivale*. Millar and Colhoun (1969) further showed that in contrast to *F. graminearum* and *F. culmorum* which are favoured by warm, dry soils, seedling disease caused by *M. nivale* was greatest in cool (< 13°C), dry (8.9% (M.W.H.C.) soil.

Colhoun (1970) reviewed the work on the epidemiology of *Fusarium* spp. in cereals. From field trials conducted at Manchester University, he concluded that the initial spread of *M. nivale* through a crop after sowing infected seed was dependent upon weather conditions during and soon after sowing. He stated that in a severe winter, the reduction in the number of surviving winter wheat plants might be as high as 87% though did not state whether those surviving seedlings showed disease symptoms or not. For spring-sown cereals, high temperatures and moist soil reduced seedling death. Colhoun (1970) concluded that air humidity at the base of plants was the most important factor in determining the occurrence of foot rot. Cereal seedlings showing stem-base lesions caused by *F. culmorum* and *M. nivale* were transferred to soil which had not carried a cereal crop for what the author described as a very long time. Foot rot only developed when air humidity at the plant base was high irrespective of soil moisture. Under these conditions, plant infections spread over what Colhoun described as a considerable distance above soil level and penetrated plant tissues deeply. Cassell and Hering (1982) studied the effects of water potential on soil-borne *F. culmorum* and *M. nivale*. Although for both pathogens, the incidence and severity of stem-base symptoms was greatest in the drier soil (-0.5 bar) compared to wetter soils (-0.1 bar), the effect was somewhat smaller for *M. nivale*. Manka (1989) considered *M. nivale* to be the most weather dependant of the cereal infecting Fusarium disease causing pathogens. Although the species has a wider temperature range over which it can develop (-5° to 22.5°C), the pathogen has a high demand for air and soil

humidity. Pronczuk and Zagdanska (1993) found that following severe winter conditions when the soil temperature fell to below –5°C for prolonged periods, infections of ryegrass caused by *M. nivale* in the following spring were severe. The growth of *M. nivale* was however restricted at temperatures below –5°C. The authors concluded that frost injured plants would be more easily penetrated by the pathogen under favourable conditions. Jenkinson (1994) studied the effect of soil moisture on the development of foot rot and sporulation of *F. culmorum, F. graminearum* and *M. nivale* on winter wheat. He found that decreasing the soil water potential from –0.15 to –60 bar resulted in an increase of both stem-base symptoms and the number of spores washed from stem-base tissues for all three pathogens. Pettitt *et al.* (1996) investigated the effect of temperature on the incidence of nodal foot rot caused by *F. culmorum* and *M. nivale.* The authors related percentage incidence of foot rot to thermal time calculated from local monthly mean air temperatures from February to July 1991 for 31 counties in England and Wales. Thermal time (in kelvin days) for the development of nodal foot rot symptoms was calculated for each county. Thermal time accounted for 53% of the variability in symptom incidence in counties where *F. culmorum* or *M. nivale* were the predominant pathogens. The authors concluded that thermal time alone was insufficient for the prediction of symptoms and other factors in particular soil water potential (Ψ soil) were important in influencing the incidence of nodal foot rot symptoms.

The expression of *Fusarium* seedling blight and foot rot symptoms are influenced considerably by environmental factors in particular soil moisture and temperature. Epidemiological studies have focused on the differences in environmental requirements of species of *Fusarium* and *M. nivale.* No information is available on the pathogenicity or environmental requirements of the two *M. nivale* sub-species as seedling blight pathogens. Such studies have been hindered until recently as the same symptoms can be caused by several fungal species.

MOLECULAR DIAGNOSTICS

The Polymerase Chain Reaction (PCR)

PCR is an enzymatic method of making multiple copies of a pre-selected segment of DNA (Kocher and Wilson, 1995). Descriptions of the principles of PCR were first made by Kleppe *et al.* (1971) however the technique was devised and named by Mullis and Faloona (1987). PCR takes advantage of a thermostable DNA polymerase (*Taq*) which carries out the synthesis of a complementary strand of DNA in a thermophilic bacterium *Thermus aquaticus*. This takes place in the 5' to 3' direction using a single-stranded template, but starting from a double stranded region and is known as the oligodeoxynucleotide 'primer' extension reaction (Taylor, 1994). The PCR employs two primers usually 18-25 base pairs (bp) in length which flank the region to be copied (Simon *et al.*, 1991). Each primer is complementary to opposite strands of the target region of DNA. The requirements of the reaction are the four deoxynucleotide triphosphates (dNTP's) (Thymine, Alanine, Cytosine and Guanine triphosphate) in equal concentrations to provide both the energy and nucleosides needed for DNA synthesis. A thermostable DNA polymerase is also required along with primers, a DNA template and a buffer containing magnesium (Taylor, 1994).

PCR takes place in three steps; template denaturation occurs when the temperature of the reaction mixture is raised rapidly to 92 - 96°C causing the double stranded template to dissociate (Taylor, 1994). The reaction mixture is then cooled, allowing the primers to anneal to their target sequences. This occurs typically at around 50°C, though is dependent upon the length and base sequence of the primer (Simon *et al.*, 1991). Finally the temperature is raised to 72°C for strand extension i.e. the addition of nucleotides in complement to the target strand sequence (Simon *et al.*, 1991). The cycle is then repeated, typically 25 - 40 times. Initially, synthesis will go beyond the complementary primer sequence, however, with each cycle the amount of DNA in the region flanked by each primer increases almost exponentially (Taylor, 1994). The quantity of DNA will continue

to increase exponentially until one of the reaction components becomes limiting or the enzyme is denatured (Mills, 1996). After twenty cycles, 2^{19} copies of the target sequence can be produced. The amplified fragment of DNA (amplicon) is confirmed as being homogenous (single size product) and of the expected size using gel electrophoresis (Kocher and Wilson, 1995). Phosphate molecules present in DNA have a negative charge which causes the DNA molecule to migrate towards a positive terminus when placed in an electric current. The charge-to-size ratio for DNA is constant although larger fragments of DNA will have a greater mass to move. For this reason electrophoresis is carried out in a matrix such as agarose gel which resists the migration of DNA. This allows smaller fragments of DNA to migrate further than the heavier larger DNA molecules (Tait, 1997). Ethidium bromide is usually added to stain the gel before electrophoresis though it can be added afterwards. In the presence of short-wave UV light, the ethidium bromide stain causes DNA to fluoresce (Ivinson and Taylor, 1994). Amplicons of between 10 bp and 2.0 kilo bases (kb) can be resolved using agarose gel though the concentration of agarose is adjusted depending on the expected product size. A 6% agarose gel was shown by Ivinson and Taylor, (1994) to resolve 56 bp and 64 bp fragments. Usually however a 2% or 3% gel is sufficient to visualise 50 bp to 500bp amplicons providing the fragments are not of very similar sizes (Ivinson and Taylor, 1994).

Restriction Fragment Length Polymorphisms (RFLP)

Until the introduction of molecular biology, there were few reliable, reproducible methods of discriminating between isolates of a fungal species (Michelmore and Hulbert, 1987). Type II restriction endonucleases recognise specific DNA sequences and cleave the DNA at these sites fragmenting a previously continuous strand of DNA. Genetic differences between fungal taxa can be distinguished by the different DNA fragment sizes after digestion using restriction enzymes (Nicholson and Rezanoor, 1993). The RFLP technique however requires relatively large amounts of DNA for digestion. Therefore, amplification

of fungal DNA prior to enzymic digestion allows the use of less DNA from the test sample and is known as PCR-RFLP. Different sized fragments (polymorphisms) detected by both RFLP and PCR-RFLP are a result of base pair additions, deletions or substitutions that vary depending on genotypic variations both between fungal isolates and species. RFLP analyses have been used to study species of *Fusarium.* For example, Nicholson *et al.* (1993) used RFLP's to investigate variation in *Fusarium* spp. causing ear blight of wheat. Of 76 single spore lines from 24 ears of wheat the authors identified four species of *Fusarium*; *F. avenaceum* (37), *F. culmorum* (33), *F. lateritium* (4) and *F. poae* (2) on the basis of spore morphology. A high degree of variation was reported between isolates of the first three species using RFLP however the two isolates of *F. poae* were found to be clonally derived. The authors reported that in 83% of cases, only a single *Fusarium* species could be isolated from each ear although separate isolates of the same species were found on different spikelets. They concluded that the infection of each spikelet is caused by separate infection unless the fungus reaches the rachis and spreads from this to infect other spikelets. Edel *et al.* (1996) was able to identify 23 haplotypes of *Fusarium* species using four restriction enzymes to cleave rDNA PCR products. Bateman *et al.* (1996) used RFLP analysis of PCR-amplified rDNA to investigate the taxonomic relationships between 34 *Fusarium* species and *M. nivale.* They compared the nuclear rDNA from a total of 120 isolates from 34 *Fusarium* spp. and *M. nivale* using RFLP analysis following PCR amplification. The authors compiled a similarity matrix for cluster analysis based on the presence or absence of 75 DNA bands. Using this they were able to estimate phylogenetic relationships between *Fusarium* spp. The authors reported little diversity between isolates of the same species and concluded that most of the species relationships they found were consistent with the current understanding of *Fusarium* taxonomy. The authors reported that *M. nivale* showed a 62.7% similarity to *Fusarium dimerum* which was the most distinct of the *Fusarium* species examined.

Random Amplified Polymorphic DNA (RAPD) Analysis

Genetic fingerprinting based upon PCR using a single short oligonucleotide primer usually 10 base pairs in length was described by Williams *et al.* (1990). The primer anneals to homologous sites in the genome of the organism being studied though no previous knowledge of the organism is required. Different primers produce characteristic profiles of amplified products for each organism (Henson and French, 1993). The arbitrary nature of such amplifications has led to the term Random Amplified Polymorphic DNA (RAPD) (Williams *et al.,* 1990). The development of RAPD analysis has allowed the identification of individual organisms, strains or even isolates. The primers used, however, will often amplify both plant and pathogen DNA, therefore, the organism under investigation must be purified or cultured prior to identification (Henson and French, 1993). Due to the variable nature of the technique, any genetic fingerprint obtained for a test organism is comparable only within that particular test. Thus genetic fingerprints obtained using RAPD analysis are compared to a control standard.

As with RFLP's, RAPD's have been used in the study of *Fusarium* species. Schilling *et al.* (1996) was able to distinguish isolates of *F. culmorum, F. graminearum* and *F. avenaceum* using RAPD analysis. RAPD techniques have also facilitated the development of specific primers without detailed knowledge of the genome of the test species. The method known as Sequence-Characterisation of RAPD fragments or SCAR, simply requires the amplification of the target species DNA using RAPD primers then sequencing a band which is common to all isolates of the species and unique to that species. Following confirmation of the uniqueness of these fragments, by Southern hybridisation, primers are then designed on the sequences obtained. This technique has been successfully employed for several pathogens of wheat including *Rhizoctonia cerealis* (Nicholson and Parry, 1996); *F. poae* (Parry and Nicholson, 1996); *M. nivale* var. *nivale* and *M. nivale* var. *majus* (Nicholson *et al.*, 1996a); *Tapesia yallundae* and *T. acuformis*

(Nicholson *et al.*, 1997b). Schilling *et al.* (1996) used the same technique to develop primers specific to *F. culmorum*, *F. graminearum* and *F. avenaceum.*

On the basis of RAPD profiles Lees *et al.* (1995) identified polymorphisms between isolates of *M. nivale* from wheat. These formed a distinct sub-group that correlated closely with a group identified as var. *majus* on the basis of conidial width. Although the remaining isolates corresponded largely to var. *nivale,* the authors failed to distinguish all isolates of *M. nivale* var. *nivale* on the basis of spore morphology. Using specific primers developed for the two *Microdochium* sub-species (Nicholson *et al.*, 1996a) provided the first molecular evidence that members of the diverse *nivale* variety of *M. nivale* did form a single group.

Quantitative PCR

PCR allows the rapid amplification of DNA regions of interest from small amounts of tissue and can therefore be used to detect specific pathogens in plant tissue. Species specific diagnostic PCR does not, however, determine the amount of pathogen DNA present. Also, as the efficiency of any PCR may be affected by several variables including untractable tube-to-tube variations, accurate comparisons between individual samples are difficult to make.

One of the first descriptions of quantitative PCR came as early as 1989 by Wang *et al.* They used competitive PCR to quantify the amount of IL-1α mRNA compared to total RNA present in the macrophage. The technique relied on competition between an unknown amount of target DNA and a constant amount of a competitor template (internal standard). The internal standard has identical primer requirements to the experimental target DNA but with a different product size (Dallman and Porter, 1994). The amount of target DNA is amplified along a dilution series containing a constant amount of the internal standard DNA. From this, a standard graph can be produced relating the concentration of target to the ratio between the target and internal standard PCR products (product ratio).

Thus the amount of experimental DNA present in subsequent samples can be derived. Changes in control variables during quantitative PCR including tube-to-tube variations affect yield of both internal standard and target template equally thus their ratios are preserved during amplification (Schnell and Mendoza, 1997).

Sensitivity

The usefulness of any diagnostic system is determined initially by its fidelity and sensitivity. The nature of the Polymerase Chain Reaction means that factors that increase the yield of a reaction may compromise the accuracy (stringency) of the amplified product. The concentrations of magnesium and dNTP's affect the efficiency of priming and extension due to alteration of the reaction kinetics at annealing and extension temperatures (Kwok *et al.*, 1990 and Huang *et al.*, 1992). Likewise, the rate at which reaction conditions change and are held at the annealing temperature affects the opportunities for mispriming and extension by molecules of otherwise idle DNA polymerase (Saiki, 1991). Non-specific PCR products may not be eliminated by optimisation of reaction conditions. The addition of tetramethylammonium chloride (TMAC) (Hung, 1990), dimethyl sulphoxide (DMSO) (Krawetz, 1989) and non-ionic detergents (Bachmann *et al.*, 1990) often influence the efficiency and enhance the specificity of PCR reactions.

When PCR reaction conditions are optimised, another factor which influences the sensitivity of PCR detection, is the copy number of target sequences within the genome. High-copy target sequences allow increased sensitivity compared to single or low copy number target sequences. Ribosomal genes and the spacers between them are desirable targets in which to develop primers due to the high number of copies of these genes present within the genome. Fungal nuclear rDNA genes are usually arranged in identical tandem repeats with 60-200 copies per haploid genome (Henson and French, 1993).

Arnheim and Erlich (1992) showed that increased sensitivity in PCR reactions could also be achieved by using a polymerase enzyme with greater thermostability such as

the Stoffel fragment of *Taq* or *Sulfololbus acidocaldarius*. The concentration of PCR inhibitors in test samples also influences PCR sensitivity. Substances which occur naturally on plant samples such as polysaccharides (Demeke and Adams, 1992), phenolics (Cenis, 1992) and humic acid (Jacobsen and Rasmussen, 1992) have all been shown to inhibit PCR reactions.

MOLECULAR DIAGNOSTICS AND INFECTIONS OF WHEAT

The identification of seedling blight, stem-base and ear blight complex fungal pathogens has traditionally relied on the visual observation of symptoms. Stem-base symptoms can be particularly difficult to diagnose during the early growth stages; *Tapesia yallundae* or *T. acuformis* (eyespot), *Rhizoctonia cerealis* (sharp eyespot), and *Fusarium* spp. foot rots can be indistinguishable (Goulds and Polley, 1990). Nicholson *et al.* (1996b) stated that the failure to identify correctly the causal agent or pathotype of disease symptoms may result in the use of an inappropriate or poorly timed disease control measure leading to yield and/or quality losses. The relative amounts of pathogen present in disease complexes in plants and seed can not be accurately determined using traditional methods (Nicholson *et al.*, 1996b). For example, Pettitt *et al.* (1993) found that when isolating on PDA from mixed wheat infections, *M. nivale* was out competed by *F. culmorum* resulting in under estimation of the former species. The use of molecular diagnostics to detect and quantify the pathogens present *in planta* allows valuable information relating to both disease complexes and single fungal infections to be obtained. Nicholson *et al.* (1996a) used quantitative PCR to investigate the colonisation of wheat seedlings cv. Mercia by *M. nivale* var. *majus* and var. *nivale*. Seedlings inoculated with var. *nivale* contained approximately 10 times more fungal DNA per mg of plant material than those inoculated with var. *majus*. In a further investigation, Nicholson *et al.*, 1996b compared the agar plate count method of assessing seed contamination by *Fusarium* spp. to quantitative PCR results for 10 seed lots. *Fusarium poae* was detected in two samples where plate counts indicated less than

1% infection by *F. poae* at harvest. *Fusarium poae* was also detected in high quantities using quantitative PCR in a seed sample that plate counts could not be made due to the high degree of contamination with the saprophyte *Epicoccum nigrum.* Nicholson *et al.* (1997a) used quantitative PCR to investigate the development of foot rot caused by *F. culmorum.* They applied spores of *F. culmorum* at three rates (500, 1000 and 5,000 conidia g^{-1} compost) and three different times (February, April and May) to compost in which winter wheat was grown. Little correlation was observed between visual disease symptoms recorded at GS75 and treatment. Quantitative PCR analyses however, showed that *F. culmorum* DNA accumulated to its highest amount when the highest amount of inoculum was added in February and April, inoculation in May even at the highest rate had little affect. Doohan *et al.* (1998) used species specific primers to investigate Fusarium ear blight infections of wheat and to compare PCR analysis to visual disease assessments in samples from a field trial where the central plot was inoculated with *F. culmorum;* plots were examined along a transect emanating from the inoculated plot to a distance of . Only in the inoculated plot and one adjacent plot did the PCR results for *F. culmorum* match visual assessments. No gradient of detection from the point of inoculation was recorded using PCR although high disease scores were obtained in plots outside those inoculated. PCR analysis indicated that this was due to *M. nivale, F. poae* and *F. avenaceum* rather than the *F. culmorum* that was inoculated. The same authors showed using PCR analysis that *M. nivale* var. *majus* was more abundant in ears than var. *nivale.* This was in keeping with earlier findings of Parry *et al.* (1995b) who showed using PCR-RFLP technique that of 91 *Microdochium* isolates taken from seed, 93% were var. *majus* and 7% var. *nivale.* Using the same technique as Parry *et al.* (1995b), Mahuku *et al.* (1998) showed that all 100 *Microdochium* isolates collected from different turfgrasses in southern Ontario belonged to var. *nivale* sub-group.

Species specific primers for Fusarium disease causing fungi allow the detection of the pathogen(s) responsible for infection before, or soon after symptoms are visible.

According to Holloman (1998) however, correlating pre-symptomatic disease levels with potential losses and linking this with established disease forecasting models remains difficult.

CONTROL OF *FUSARIUM* SEEDLING BLIGHT

Chemical Control

Though introduced primarily to control bunt, organomercury compounds were among the first chemical seed treatments used for the control of *Fusarium* seedling blight. Experimental work by Bolley (1897), Farrer and Sutton (1905) and Remy and Vasters (1914) led to the introduction of 'Uspulun' known commonly as quicksilver in 1914. Machacek and Greaney (1935) tested the ability of several compounds to disinfect the seeds' surface including nickel sulphide, iodine-infusorial earth, substances containing copper carbonate and also organo-mercury compounds. Organo-mercury treatments were the most effective, significantly increasing the yields of wheat sown in soil heavily infected with *F. culmorum.* Richardson (1974) used field trials to investigate the effects of organmercurial seed treatments on the growth and yield of oats grown from seed infected with *M. nivale.* Yield was increased through treatment in 46 out of 61 seed batches tested with a mean increase of 6.7% over the untreated. The author attributed the improvement to increased plant populations as a result of improved seedling emergence. Bateman (1977) investigated the effects of phenyl mercuric acetate (PMA) on germination and stem-base disease. Rates of PMA between 0.5 and 3.0 μg of mercury per seed significantly increased germination and reduced stem-base disease when sown in soil infected with *M. nivale* and *F. culmorum.* In later experiments, Bateman (1983) showed that PMA provided only limited control of deep-seated infections by *M. nivale.* Inoculum located in the epidermis and to a lesser extent the inner pericarp was controlled by PMA but not that located in the testa, endosperm or seed embryo.

Organomercurial seed treatments dominated the UK market for over fifty years (Paveley *et al.*, 1996) and in 1977, 95% of all UK cereal seed was treated with organomercury fungicides (Steed *et al.*, 1979). Concerns over the toxicity of mercury containing agents led to an EC council directive (79/117/EC) in 1979 prohibiting the use of mercury in agriculture (Anon., 1979) although a derogation allowed their continued use in the UK until 1992 (Paveley *et al.*, 1996).

Due to the withdrawal of organomercury seed treatments several alternatives particularly from the demethylation inhibiting (DMI) and methyl benzimidazole (MBC) groups of fungicides were introduced. These had previously been used as an alternative to mercury based compounds. Wainwright *et al.* (1979) showed that pre-emergence seedling death caused by seed-borne *M. nivale* was controlled more effectively when fuberidazole (an MBC fungicide) was used in mixture with triadimenol (a DMI fungicide). The widespread use of MBC fungicides for the control of stem-base disease complex fungi led to the development of resistance and the subsequent loss of effective control of *M. nivale.* Hartke and Buchenauer (1985) reported that seed treatments with the MBC fungicides carbendazim, thiabendazole and fuberidazole did not prevent snow mould in Germany due to resistant strains of *M. nivale.* These findings were supported by those of Locke *et al.* (1987); in a survey of benomyl resistance in *Fusarium* spp. in England and Wales in 1986 they found that of 581 isolates of *M. nivale* isolated from infected stem-bases 92.1% were resistant to benomyl. The authors detected no reduction in the sensitivity towards benomyl of isolates of *F. avenaceum, F. culmorum* or *F. poae.* Scheinpflug and Duben (1988) stated that an MBC should not be the only ingredient used in a seed treatment for the control of *M. nivale* and concluded that a seed treatment should be supplemented by a substance to which *M. nivale* is not resistant. Similarly, Wainwright and Linke (1987) concluded that the MBC fungicide fuberidazole should be added to the DMI fungicide tebuconazole to improve the control of *M. nivale.*

More recently, new compounds formulated as seed treatments have become available which show activity against seedling blight. The isolation of pyrrolnitrin from *Pseudomonas pyrocinia* was first reported in 1965 by Arima *et al.* Pyrrolnitrin formed the lead molecule around which the phenylpyrrole fungicides are based. Pyrrolnitrin is highly photolabile, empirical optimisation around this lead structure led to the synthesis of two, more stable derivatives, fenpiclonil (1982) and fludioxonil (1984). Biological screening showed both fungicides to be highly active against a range of *Deuteromycete, Ascomycete* and *Basidiomycete* fungi when applied as either a seed treatment or foliar spray (Leadbitter *et al.*, 1994). Fenpiclonil was introduced in 1988 under the trade name 'Beret' and fludioxonil was released commercially in 1995 as 'Beret Gold'. Both are applied as seed treatments for the control of *Fusarium* spp., *Gibberella* spp., *M. nivale*, *Tilletia* spp., *Helminthosporium* spp. and *Septoria* spp. (Anon., 1999). The effects on stem-base seedling blight of using Beret Gold as a fungicide seed treatment can be seen in Figure 1.5.

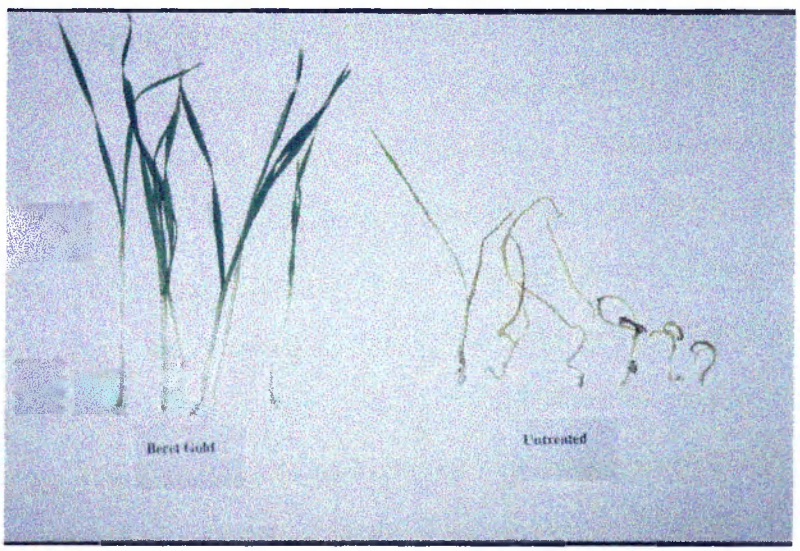


Figure 1.5. Seedlings produced from *M. nivale* infected, untreated seed (right) and *M. nivale* infected, Beret Gold treated seed (left).

The mode of action of phenylpyrrole fungicides is somewhat unclear. Leroux *et al.* (1992) considered the mode of action to be similar to that of the dicarboximides; the accumulation of free radicals caused by the blockage of electron flow from NADPH to cytochrome-c-reductase. Jespers (1994) studied the mode of action of fenpiclonil on

Fusarium sulphureum concluding that the toxic action was caused by blockage of transport-associated glucose phosphorylation.

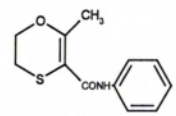
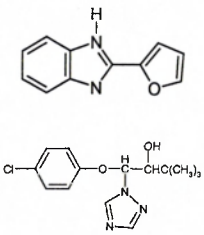
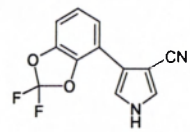
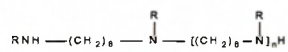
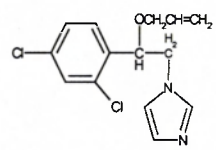
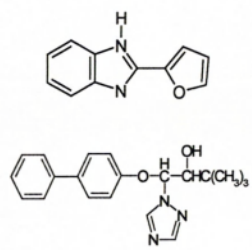
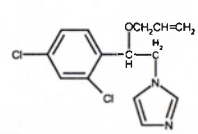
Other fungicide seed treatments, which are effective against seedling blight, include bitertanol (DMI) (Morris *et al.*, 1994) and guazatine (guanidine) (Jackson *et al.*, 1973). Conflicting reports are available on the effectiveness of the carboxamide seed treatment carboxin. Uoti (1979) observed little effect on disease under glasshouse conditions when artificially inoculated seed was treated with the fungicide. Scheinpflug and Duben (1988) concluded that the action of carboxin on *M. nivale* was insufficient for its control. Seed treatments currently recommended for the control of fusarium diseases are listed in Table 1.1.

Alternative seed treatments

Early attempts at controlling seedling blight causing organisms involved soaking seed in hot water (Westerdijk, 1909) or exposing contaminated seed to 'dry heat'. The latter method was shown by Naumov (1917) to successfully control *F. graminearum*. Atanasoff and Johnson (1920) could not reproduce the findings of Naumov though they did achieve successful control by heating the seed to 100°C for 30 hours. The germination of seed treated in this way was however reduced by 50%.

More recently, Linder and Jahn (1998) investigated the effects of electron-beam irradiation on *F. culmorum* and *M. nivale* present in artificially infected wheat seed. The authors noted that seed-borne *M. nivale* was reduced to undetectable levels by an irradiation dose of 7 kGy whereas the field emergence of irradiated seeds infected by *F. culmorum* was considerably increased. The authors failed to report the lethal electron-beam dose against *F. culmorum* or the field performance of irradiated *M. nivale* infected seed.

Table 1.1. Seed treatments recommended for the control of Fusarium diseases in the UK (Anon, 1999).

Product Name	Active Ingredient(s)	Structure(s)	Fusarium disease controlled/ Crop	Chemical Group
Anchor	Carboxin + Thiram		Fusarium foot rot and seedling blight Barley, oats, rye, wheat and triticale	Carboxamide + Dithiocarbamate
Baytan Flowable	Fuberidazole + Triadimenol		Foot Rot Barley, oats, rye, triticale and wheat.	Benzimidazole Conazole
Beret Gold	Fludioxonil		Foot rot Fusarium foot rot, seedling blight Snow mould Spring barley, winter barley, winter wheat.	Phenylpyrrole
Panoctine	Guatazine		Fusarium foot rot and seedling blight (reduction)	Guanidine
Panoctine Plus	+ Imazalil		Barley, oats and wheat	Conazole
Sibutol	Fuberidazole + Bitertanol		Foot rot and Seedling blight winter wheat (reduction). Fusarium root rot in winter oats.	Benzimidazole Triazole
Stryper Fungaflor 100 SL	Imazalil		Brown foot rot Winter and spring barley	Conazole

Biological Control Agents

The first reports on inhibition of seedling blight causing fungi by soil microflora came in the early 1930's. Since then much of the work on the biological control of Fusarium diseases of cereals has focused on the application of organisms showing antagonistic effects *in vitro* either to soil or by treatment of the seed. Bisby *et al.* (1933) used agar plate tests and pot experiments to show the suppression of *F. culmorum* by *Trichoderma lignorum*. *Fusarium culmorum* was rapidly outgrown and destroyed by *T. lignorum* on agar plates. In pot experiments, seedling blight was evident in wheat seedlings grown in soil infested with *F. culmorum* alone. No symptoms were observed on seedlings grown in soil where *T. lignorum* was applied either singly or in combination with *F. culmorum*. Tveit and Wood (1955) investigated the ability of antagonistic species of *Chaetomium* to control Fusarium seedling blight of oats caused by *M. nivale*. Most were ineffective however, some isolates of *C. cochliodes* and *C. globosum* gave disease control comparable to standard organomercurial seed dressings assessed as percentage emergence and percentage healthy seedlings, these results were confirmed through field trials. The authors also showed that *C. cochliodes* persisted in unsterile soil for over 10 months. Millar and Colhoun (1969) isolated five fungi antagonistic to *M. nivale* from two soils they examined. *Gliocladium roseum* was the most effective of these fungi in controlling the incidence of disease when artificially inoculated wheat seed was sown in soil in which the antagonistic fungus was growing. As little was known about the relative frequencies of surface and internal infection of wheat seed with *M. nivale* the authors suggested that it would not be possible to assess the effects of soil microflora in controlling disease in the field. Tahvonen and Avikainen (1990) investigated the control of *F. culmorum* using the biofungicide 'Mycostop' as a cereal seed dressing. The active organism in Mycostop-*Streptomyces griseoviridis* is believed to act by the secretion of antibiotic substances. Control of *F. culmorum* was achieved in agar plate tests though, variable results were observed in pot and field experiments and the control observed was significantly less than

when the chemical mercury dust Ceresan was applied. Lahdenperä *et al.* (1991) reported that Mycostop controlled seedling blight of wheat from both artificial and natural infection caused by *F. culmorum*. The authors reported that yield was significantly increased when compared with the un-named control. In contrast Tahvonen and Avikainen (1990) concluded that under field conditions control would not exceed that achieved with chemical treatment and that Mycostop was not sufficiently active against pathogens which penetrated the seed. The development of biological control agents into the fungicide market is fraught with problems. Antagonists that exhibit good activity in laboratory experiments or relatively sterile environments such as composts or soils that have been sterilised or fumigated are often unable to compete under field environments containing a complex microflora. Also, the bulking up of sufficient inoculum required for commercial use is often not cost-effective, this combined with the fact that agents are often unable to survive without the pathogen as a substrate means that the agent must be regularly applied again increaseing costs.

AIMS

The aims of this study were:-

i) To develop PCR techniques which would allow the detection and quantification of *M. nivale* var. *nivale* and var. *majus* inoculum *in planta*.

ii) To compare PCR based methods for determining the severity of seed-borne *M. nivale* contamination of seed lots to agar plate count techniques and determine their relationship with seedling blight disease.

iii) To examine annual and region differences in the amount of seed-borne *M. nivale* var. *nivale* and var. *majus* inoculum and determine the pathogenicity of isolates of *M. nivale* var. *nivale* and var. *majus* as seedling blight pathogens of wheat.

iv) To examine the effectiveness to fungicide seed treatments against *M. nivale* var. *nivale* and var. *majus* (a) *in vitro*, (b) under a range of controlled temperatures (c) under field conditions.

CHAPTER 2

GENERAL MATERIALS AND METHODS

MATERIALS AND METHODS

CULTURE OF HOST WINTER WHEAT (*TRITICUM AESTIVUM* L.)

Seedling growth media and sowing of seed

For non-field seedling experiments, John Innes No. 2 potting compost was used. Compost was passed through a 5 mm sieve and autoclaved at 121°C 103.4Kpa for 60 min three times. The compost was allowed to cool completely between each autoclave cycle. Seeds were planted to a depth of 2 cm. Containers used were either (i) Plastic tray; (215 x 155 x 45 mm) (ii) Plastic pot; 70 x 70 x 80 mm (Desch Packaging, Beuningen, Holland), (iii) Glass jar - wide, screw neck; 60 x 60 x 120 mm (Fisons, UK). Asceptic operations were performed in a sterile laminar air flow cabinet, media and distilled water were sterilised in an autoclave at 121°C and 103.4 Kpa for 20 min.

Seed surface sterilisation

Seed was placed in a sodium hypochlorite solution (1.2 % available chlorine), containing 0.05 % Tween 20 (Sigma, UK) for 3 min to eliminate any surface contaminants. The seed was rinsed in three changes of sterile distilled water (SDW) and placed in an open Petri dish (Sarstedt, UK) in a laminar air flow to dry.

Determination of percentage M. nivale *and* Fusarium *spp. infection in seed batches*

Surface sterilised seed was placed crease downwards onto the surface of potato dextrose agar (PDA) (Merck, Germany) (Appendix 1) amended with the antibiotic streptomycin sulfate (Sigma) (130 $\mu g\ ml^{-1}$ agar). Five seeds were placed into each of forty plates and incubated at 20°C for 7-14 days under near ultra violet light (NUV) (12 h photoperiod). Colonies of *M. nivale* were identified by the production of salmon pink mycelium (Brayford, 1989) whereas colonies of *Fusarium* spp. were identified by the production of deep red cultures (Brayford, 1989). The number of seeds producing colonies

of each type was recorded and the incidence of each within the seeds tested was calculated in terms of percentage infection.

CULTURE OF PATHOGEN

A list of all the isolates used in this study is given in Appendices 2 and 6.

Isolation of Pathogens from Seed and Production of Single Spore Cultures

Colonies of *M. nivale* emanating from surface sterilized seed after incubation on PDA were isolated to a fresh PDA plate by the removal of a small piece of mycelium. Plates were incubated at 20°C for 7-14 days under NUV, 12 h photoperiod to induce sporulation. A small quantity of spores was removed from each culture using a sterile microbiological loop and mixed in 1 ml SDW, spread onto a PDA plate and incubated at 20 °C. After 48 h, germinating, single conidia were identified under a binocular field microscope and transferred to a fresh PDA plate and incubated at 20 °C prior to species identification. A list of all isolates used in this study and their origin is given in Appenices 2, 6 and 7.

Storage of fungal cultures

A 5 mm mycelial plug was taken from the edge of an actively growing culture and transferred to a sterile universal bottle containing approximately 10 ml of synthetic nutrient agar (SNA) (Appendix 1) (Nirenberg, 1976) which was allowed to set at an angle of approximately 45°. The cultures were incubated at 20°C for 7 days to allow the culture to develop before being stored at 4 °C. Isolates were sub-cultured onto fresh SNA slopes every 3 months.

Production of spores and spore suspensions

Sub-cultures of each isolate were made by transferring a small amount of mycelium from an SNA slope onto plates of PDA using a microbiological loop. The plates were incubated at 20°C under NUV, 12 h photoperiod for 7-14 days. Mycelium and conidia

were washed from the surface of sporulating cultures using 15 ml of SDW by gentle rubbing with a sterile glass rod. The suspension was filtered through two layers of sterile muslin to remove hyphal fragments. Spore concentrations were determined under a light microscope using a haemocytometer (Weber Scientific International Ltd, UK) and adjusted to the required concentration using SDW.

Glasshouse production of infected grain

Five wheat seeds cv. Cadenza were planted into plastic pots (15 cm diameter) containing compost (John Innes No. 2) and maintained at 16°C and a 16 h photoperiod. At mid anthesis (GS65) plant ears were inoculated with a conidial suspension containing $1x10^5$ spores ml^{-1} at a rate of 2 ml per ear of an isolate or isolates of either *M. nivale* var. *nivale* or *M. nivale* var. *majus* using a pressurised hand-held spray bottle. Where a mixture of isolates was used, equal proportions of each isolate were added to make up the final concentration of $1x10^5$ spores ml^{-1}. Self sealing plastic bags were placed over the ears of each inoculated pot for 48 h after inoculation to aid infection. Plastic bags were removed for 24 h, inoculation was repeated and plastic bags replaced for a further 48 h. Ears were cut from the plants at GS92 and ears were threshed using a Hege 16 single ear thresher (Hans-Ulrich Hege GmbH & Co, Waldenburg, Germany).

Assessment of seedling blight diseases symptoms

The severity of seedling blight symptoms on emerged seedlings was assessed using a 0-3 scale for healthy, slight, moderate and severe symptoms (Table 2.1). A disease index was produced (total disease score divided by total number of plants) which was divided by three to give a value between 0-1 and arcsine (Asin) transformed before analysis. Statistical analyses were performed using Genstat® Version 4.1 for Windows (Lawes Agricultural Trust, UK).

Table 2.1 Assessment key used for visual assessment of disease.

Scale	Description
0 (healthy)	No visible symptoms.
1 (slight)	Stem base browning present- does not girdle stem-base.
2 (moderate)	Stem browning present which completely girdles stembase.
3 (severe)	Stem browning, completely girdles stembase, dark brown in colour and/ or extends up stembase to above ground parts of seedling.

Equation 1. Equation used to produce disease index data where n = number of seedlings in category

ArcSin transformation of :-

$$\sqrt{\left\{ \frac{\frac{(n * 0 \text{ healthy}) + (n * 1 \text{ Slight}) + (n * 2 \text{ moderate}) + (n * 3 \text{ severe})}{\sum n}}{3} \right\}}$$

MOLECULAR METHODS

DNA Extraction - fungal colonies

A small amount of mycelium was removed from a fungal colony using a sterile microbiological loop and placed into a sterile 1.5 ml Eppendorf tube. Chelex carbon buffer (0.25 ml) (Appendix 1) was added and the mycelium was crushed again, using a sterile micropestle. Tubes were vortexed for 10 s then boiled for 10 min and left to cool before being centrifuged at 12,000 *g* for 15 min. The supernatent (100 µl) was removed to a fresh tube and diluted two fold in TE buffer for use in PCR reactions.

DNA Extraction - broth cultures

Where a larger quantity, or purer fungal DNA was required for use over a longer period of time, fungal DNA was extracted from fungal broth cultures. Potato dextrose broth (PDB) (Difco Laboratories, USA) (Appendix 1) (50 ml) was dissolved in SDW in a

250 ml conical flask (Sterilin, UK), sealed and sterilised in an autoclave (121°C 105.4 KPa for 20 min). A small amount of fungal mycelium from isolates of known species was added to the broth using a sterile microbiological loop. Flasks were incubated at 20°C in an orbital refrigerated incubator (Sanyo, UK) at 150 rpm. After one week, the liquid phase was discarded and the solid mycelium was retained and dried on a sterile filter paper in a laminar air flow. A small piece of mycelium was placed in a 1.5 ml tube, 0.6 ml of CTAB extraction buffer was added and the mycelium was crushed using a sterile micropestle. Tubes were frozen and thawed using a dry-ice-ethanol bath and 65°C water bath four times, after the final heating, the mycelium was crushed using a sterile micropestle and incubated at 65°C for 1 h. Chloroform (0.6 ml) was added and the tubes were vortexed and centrifuged for 15 min before 0.5 ml of the aqueous phase was removed to a fresh 1.5 ml Eppendorf tube. Isopropanol (0.4 ml, 100 %) was added and the tubes were mixed gently for 2 min and then incubated for 30 min at 20°C to precipitate DNA. Tubes were centrifuged at 6000 *g* for 15 min, the aqueous phase discarded and 1 ml 44 % isopropanol was added, the tubes were centrifuged at 6000 *g* for 15 min, the aqueous phase discarded and the process was repeated. The DNA was air dried in a laminar airflow cabinet before being dissolved in TE buffer (Appendix 1).

DNA Extraction - wheat seedlings and plants

Plant material for extraction was placed in 50 ml kartell tubes (Fisher, UK) and frozen for 16 h before being freeze dried in a Modulyo freeze drier (Edwards, UK). After one week, tubes were removed from the freeze drier and four sterile stainless steel ball bearings (one 22 mm and three 8 mm diameter) were placed in each tube and shaken using a soil mill (Griffin, UK) for 1-2 h. The powdered, freeze dried, plant material was transferred to a sterile 50 ml centrifuge tube (Sarstedt) and weighed. A CTAB (Appendix 1) based extraction buffer was added at a rate of 30 ml for samples whose weight exceeded 1 g and 10 ml for those less than 1 g. Tubes were incubated at 65°C for 1 h then allowed to cool to

room temperature before adding 5 M potassium acetate (BDH Laboratory Supplies, UK) at a rate of one third of the volume of CTAB extraction buffer added. Tubes were incubated at –20°C for 16 h. Tubes were thawed and then centrifuged at 2500 *g* for 15 min, before 1.3 ml of the supernatant was removed to a sterile 1.9 ml Eppendorf tube, 0.6 ml of chloroform (BDH) was added and the tubes mixed gently for 1 min and centrifuged at 12000 *g* for 5 min. The supernatant (1 ml) was removed to a sterile 1.9 ml Eppendorf, 0.8 ml of 100 % isopropanol (BDH) was added and the tubes were mixed gently for 2 min then incubated at 20°C for 30 min to precipitate DNA. The tubes were centrifuged at 6000 *g* for 15 min to form a solid DNA pellet. The aqueous phase was discarded and 1 ml of 44 % isopropanol was added and the tubes centrifuged at 6000 *g* for 15 min. The supernatent was discarded and the process repeated. The DNA was air dried for 16 h at 20°C in a laminar air flow cabinet before being dissolved in TE buffer (Appendix 1).

DNA Extraction - seed

Approximately 14 g of wheat seed was placed into a plastic pouch made from an A4 photocopy acetate folded in half and sealed around two edges (Lloyd Paton Ltd, UK). Grain were crushed using a 2.5 lb masonary hammer until no whole seeds remained (Figure 2.1), 10 g of the crushed seed was then weighed into a sterile 50 ml tube (Sarstedt), 30 ml of CTAB extraction buffer was added and the tubes were incubated at 65°C for 16 h. The remainder of the extraction procedure was identical to that for plant material.

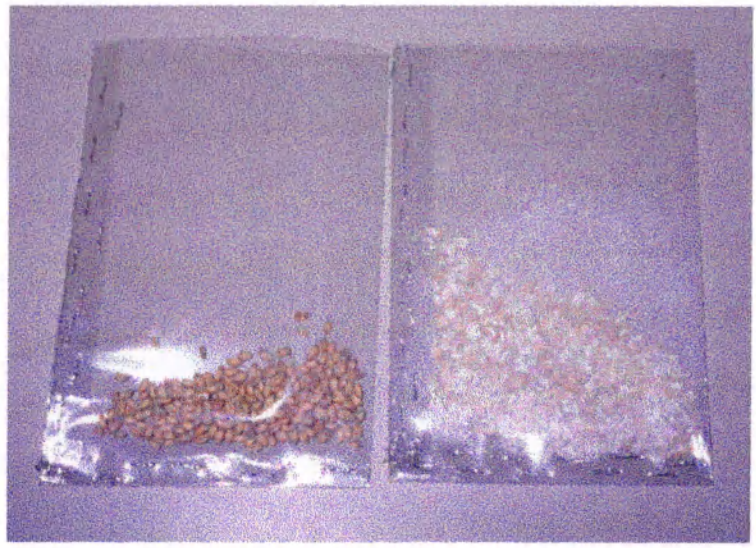


Figure 2.1. Plastic pouch containing uncrushed (left) and crushed (right) grain.

Determination of the concentration of extracted DNA

Crude DNA extractions were diluted 1:20 in TE buffer to a final volume of 0.2 ml. The sample (0.09 ml) was placed in a micro-cuvette previously rinsed with two changes of SDW and five changes of TE buffer. Absorbance was measured using a DUK400 spectrophotometer (Beckman Instruments, USA) at 260, 280, and 320 nm. The final concentration of DNA in each sample was determined using the Warburg Christian co-efficient (Anon, 1992). Samples were diltued to a concentration of 40 ng μl^{-1} for use in PCR reactions.

PCR Amplification and agarose gel electrophoresis

Diagnostic PCR reactions were performed in 25 µl volumes and contained 5 µl of the diluted DNA sample. Quantitative PCR reactions were performed in 50 µl volumes and contained 10 µl of the diluted DNA sample and 10 µl of the appropriate internal standard. A PCR reaction buffer was added (Appendix 3) along with 100 nM each of the relevant forward and reverse primers (Appendix 4), and one unit of Super *Taq* polymerase (Kramel Biotech Ltd., UK) per 50 µl reaction.

Amplification was performed in a PTC-100 thermal cycler (MJ Research Inc., USA), the PCR programme used for amplification varied depending on the primer pair used (Appendices 4 and 5). Amplification products (10 µl) were electrophoresed through agarose gels (2.0 % w v^{-1}), prepared using TAE buffer and containing 0.05 mg ethidium bromide per 100 ml TAE buffer (Appendix 1).

Analysis of PCR products

Amplicons were visualized following gel electrophoresis using a Gel Doc 1000 flourescent gel documentation system (Bio-Rad Laboratories Ltd). For quantitative PCR reactions, the intensity of amplicons was determined by analysis of unsaturated images using Molecular Analyst 1.5 software (Bio-Rad). The ratio in intensity of fungal to internal standard PCR product was determined for each sample and the amount of fungal DNA determined by interpolation from the relevant standard curve. Any sample with too much target DNA was diluted and quantification repeated.

CHAPTER 3

DEVELOPMENT OF DIAGNOSTIC ASSAYS FOR THE DETECTION AND QUANTIFICATION OF *M. NIVALE* VAR. *NIVALE*, VAR. *MAJUS* AND *FUSARIUM* SPP. DNA

INTRODUCTION

Several fungal pathogens are able to cause Fusarium seedling blight infections of wheat however identification of the species responsible is not possible in mixed infections from symptoms alone. As several fungal species can be present within samples which are infected with seedling blight pathogens, the selective isolation of the pathogen is often difficult. Selective media for the isolation of *Fusarium* spp. were described by Nash and Snyder (1962) and Tio *et al.* (1977). Pettitt *et al.* (1993) showed that the incorporation of benomyl into potato dextrose agar significantly improved the estimation of *M. nivale* from infected stem bases. By controlling the faster growing and benomyl sensitive *Fusarium* spp., the incidence of *M. nivale* detection was improved. An average of 7% of isolates of *M. nivale* were benomyl sensitive. They reported that in mixed infections where benomyl sensitive strains of both *M. nivale* and *Fusarium* spp. were present, *M. nivale* was much less likely to be isolated on either benomyl amended or unamended agar. They stated that the solution to this diagnostic problem would be the development of molecular techniques for pathogen identification.

Taxonomic methods used for the classification of species of *Fusarium* have in the past yielded conflicting results. The genus is divided into sections on the basis of morphological characteristics. The 16 sections within the genus *Fusarium* described by Wollenweber (1931) were later simplified by Snyder and Hansen (1945) into individual species each with separate biological forms. Booth (1971) used the production of polyblastic conidiogenous cells as a taxonomic distinction for the section *Arthrosporiella* however, Nelson *et al.* (1983) did not use this feature as a section criterion. The identification of many *Fusarium* species and groups can be problematic due to the wide variation in morphological and non-morphological characteristics used to separate members of the genus, a problem reported by Snyder and Hansen (1945). Several methods are described for the identification of *M. nivale* based on morphological characteristics (Booth, 1971, Gerlach and Nirenberg, 1982 and Brayford, 1989).

The identification of pathogens has in the past relied on the isolation of the organism from infected plant material, and axenic culture techniques followed by identification of the causal agent on the basis of colony or fungal structure morphology. After growth in culture, spores are examined microscopically and classified according to a number of criteria such as size, shape and presence or absence of distinctive features. Brayford (1989) reported problems in the identification of *Fusaria* to species level directly from isolation plates as colonial and spore morphologies are often atypical. Windels (1991) reported that *Fusarium* spores produced by cultures grown on carnation leaf agar (CLA) were more uniform in size and shape and phenotypic variation was reduced compared to growth on PDA, which often produced atypical spores. She suggested CLA should be used to produce microscopic features needed for identification whereas PDA should be used to produce culture characteristics. Such criteria may however overlap between species confounding further the identification of *Fusarium* to species level. Lees *et al.* (1995) reported that the spore morphology of isolates of *M. nivale* grown on PDA was a continuous variable which overlapped between isolates conforming to variety *majus* and variety *nivale* on the basis of RAPD profiles. In contrast to Windels (1991), Nelson (1991), concluded that observations of cultures grown on selective media tended to emphasise differences such as size and shape of macroconidia within *Fusarium* spp. rather than similarities and exaggerate minor differences the result being finer separations at the species, variety and form levels.

To facilitate the identification of *Fusarium* species, further criteria have been utilised. The use of gel electrophoresis of total soluble proteins as a tool in *Fusarium* taxonomy was investigated by Glynn and Reid (1969), the authors reported that banding patterns for isolates of *Fusarium oxysporum* varied with environmental conditions. Lauren *et al.* (1992) used production of the trichothecenes nivalenol and deoxynivalenol by isolates *F. graminearum* to distinguish them from isolates of *F. culmorum* and *F.*

crockwellense which produced mainly nivalenol. Marasas (1984) used the inability to produce tricothecenes to distinguish isolates of *M. nivale.*

The taxonomic systems described so far are based on phenotypic characteristics of the fungal species and therefore may vary depending on abiotic factors affecting the production of the identifying feature. In a review of *Fusarium* taxonomy, Nelson (1983) stated that many of the characters originally used by Wollenweber and Reinking (1935) to separate species, varieties and forms were not stable and could be altered by growing cultures on various media and varying environmental conditions.

The use of restriction fragment length polymorphisms (RFLP's) as molecular markers for the genetic analysis of phytopathogenic fungi has been exploited for studies on *Fusarium* spp. RFLP analysis of mitochondrial and rDNA has been used to examine the relationships among *Fusarium* spp. (Bateman *et al.*, 1996, Edel *et al.*, 1996), to analyse vegetative compatibility groups and pathogenicity in *Fusarium oxysporum* f. sp. *dianthi* (Manicom *et al.*, 1990), and to analyse variation in *Fusarium* species causing ear blight of cereals (Nicholson *et al.,* 1993). Lodolo *et al.* (1992) used a clone of the *Neurospora crassa* rDNA as a probe to identify RFLPs within rDNA genes of toxin-producing strains of *Fusarium.*

Mule *et al.* (1997) reported that 28S rDNA sequences of 16 species of *Fusarium* showed greater correlation to trichothecene production than classification systems based on morphology. The authors concluded that the use of rDNA sequences in conjuction with mycotoxin production potential could provide a basis for identification of uncertain and atypical toxigenic *Fusarium* strains.

Advances in molecular biology have allowed the development of accurate methods for pathogen identification and detection and have been effectively applied through the use of species specific primers. The development of species specific primers for plant pathogenic fungi has traditionally centred on the use of RAPD profiles to identify a band common to that species which is confirmed as being unique by hybridisation assays.

Sequence analysis of such bands followed by the design of primers at points in the sequence which are common to all isolates yet heterologous to other species of fungi has led to the development of primers specific to several fungal pathogens of wheat. The technique, sequence characterisation of amplified RAPD's (SCAR), allows primers to be designed for species of fungi with little or no information as to the genetic composition of the organism in question. Species specific primers designed in this way are based usually on unknown genomic regions and thus regions of unknown copy number. The DNA copy number is an important determinant of the sensitivity of a PCR-based assay (S. G. Edwards pers. comm). SCAR-based primers may therefore result in a less sensitive assay compared to an assay based on primers designed to amplify regions of known high copy number DNA such as rDNA.

The ribosomal genes and in particular the spacers between them have proved popular targets for both molecular detection and phylogenetic studies as they occur in high copy numbers and possess both conserved and variable regions (Henson and French, 1993). The ribosomal genes themselves are well conserved due to the selection pressures required to maintain functionality, however the non-encoding spacers in between these genes are usually conserved at the species level but variable in higher taxa (Bruns *et al.*, 1991). Primers which allowed the amplification of part of the small and large rDNA sub-units as well as the complete sequences of the 5.8S rDNA sub-unit and ITS 1 and 2 regions in fungi were described by White *et al.* (1990). These primers have allowed the identification of polymorphisms within rDNA which has facilitated the design of species specific primers. The ITS1 and ITS2 primers described originally by White *et al.* (1990) were used by Johanson (1994) to amplify a section of rDNA from isolates of the Sigatoka leaf spot fungi *Mycosphaerella musicola* and *M. fijiensis*. The authors were able to develop primers specific to each pathogen based on the differences within the ITS1 region of rDNA. Edel *et al.* (2000) used the forward primer (ITS1) described by White *et al.* (1990) in combination with a reverse primer described by Guadet *et al.* (1989) to amplify

parts of the 28S rDNA from isolates of *Fusarium oxysporum*. Analysis of the amplified fragments revealed variable nucleotides between the sequence of *F. oxysporum* and those of other species. The authors used these differences to design primers specific for the pathogen. The technique has also been successfully employed in the design of primers for *Verticillium dahliae* and *V. albo-atrum* (Nazar *et al.*, 1991) *Serpula lacrymans* (Schmidt and Moreth, 2000) and *Rhizoctonia solani* (Salazar *et al.*, 2000). Although ITS regions of rDNA are generally conserved at the species level, Ward and Gray (1992) were able to distinguish separate sub-species from ITS sequences of isolates within the *Gaemannomyces-Phialophora* complex and O'Donnell *et al.* (1992) reported extensive divergence between ITS sequences of strains of *Fusarium sambucinum*.

In recent years, the development of sequence databases such as the European Molecular Biology Laboratory (EMBL) combined with the rapid expansion of the World Wide Web has facilitated the exchange of DNA sequences for many eukaryotes. The information available in such databases allows plant pathologists to obtain sequences relating to a specific genomic region of a particular species or taxonomic group of species. Sequence information obtained in this way can be used to identify polymorphisms between individual species or taxonomic groups in a range of isolates from a variety of geographical sources. This in turn allows primer sets specific to the test organism or group to be designed with minimal laboratory work or duplication of sequencing work already performed in another laboratory.

Chapter Objectives

The objectives of the work presented here were (i) To develop assays, which will allow the quantification of *M. nivale* and *Fusarium* spp. by competitive PCR using primers based on the ITS region. (ii) To develop quantitative PCR assays for the two sub-species of *M. nivale*; *M. nivale* var. *nivale* and *var. majus* based on the same genomic region.

MATERIALS AND METHODS

M. NIVALE AND *FUSARIUM* SPP. SPECIFIC PRIMERS

Primer sequences for the specific amplification of either *M. nivale* or *Fusarium* spp. were obtained from Dr. Jim Beck, Syngenta Agribusiness Biotechnology Research Inc. (US Patent No. 5,827,695) and are termed JBM and JBF respectively (Appendix 4). These primers target the ITS regions of rDNA for these fungi. Alignments of the rDNA sequences used for the design of the *M. nivale* specific primers are given in Figure 3.1. Internal standards were constructed for primers JBF and JBM for use in competitive PCR assays (S.G. Edwards, unpublished).

M. NIVALE VAR. *NIVALE* AND VAR. *MAJUS* SPECIFIC PRIMERS

Analysis of rDNA

Nucleotide sequences for the rDNA of isolates of *M. nivale* var. *nivale* and var. *majus* were obtained from Syngenta Agribusiness Biotechnology Research Inc. (SABRI) details of the isolates used are given in. Sequences were aligned using Clustal W (Thompson *et al.*, 1994) sequence alignment program at www.workbench.sdsc.edu. A forward primer was designed for each sub-species at conserved points, annealing temperatures were calculated using 'Primers' at www.williamstone.com (accessed Jan/2000) and matched to a common reverse primer (NGMic/R) (Appendix 4). The PCR reaction conditions and PCR were as described in Chapter 2 (page 41). The PCR Program A, as described in Appendix 5 was used with the exception that annealing temperatures of 54 – 62°C were tested in separate reactions in order to optimise stringency.

Elongation factor 1-α gene

Amplification of the EF-1α gene in *M. nivale*

No sequence information was available for the EF-1α encoding gene within *M. nivale*, consequently, nucleotide sequences encoding the EF-1α gene for several filamentous fungi were retrieved from the EMBL sequence database (Species/ accession numbers: *Puccinia graminis*/ X73529, *Schizophyllum commune*/ X94913, *Aureobasidium pullulans*/ U19723, *Trichoderma reesei*/ Z23012). Sequences were aligned using Clustal W (Thompson *et al.*, 1994) at www.workbench.sdsc.edu and primers were designed where the sequences were most similar. The sequences showed few points conserved between all the fungi tested, therefore, degenerate primers were designed. This 'population' of primers would increase the likelihood of the primers annealing to the EF-1α gene in isolates of *M. nivale*. Combinations of three forward (EF1/F, EF2/F and EF3/F) and two reverse (EF1/R and EF2/R) primers obtained (Appendix 4) were tested to determine which primer pairs gave the expected product sizes when DNA from isolates of *M. nivale* var. *majus* and var. *nivale* was amplified. Reaction conditions were as described for diagnostic PCR with PCR program A (Appendix 5) with an annealing temperature of 52°C.

Optimisation of PCR reaction conditions using Taguchi methods

The major factors that influence PCR stringency and yield (concentrations of magnesium, primers and template DNA) were adjusted (Table 3.1) according to Taguchi methods described by Cobb and Clarkson (1994) in order to optimise the yield of the desired EF-1α gene. In all other PCR reactions, reagents were as described in Chapter 2 (page 41).

Table 3.1. Concentrations of $MgCl_2$, primers and template DNA used.

Treatment	Concentration of $MgCl_2$ (mM)	Concentration of each primer (Units)	Concentration of template DNA (ng μl^{-1})
1	1.5	1	1
2	1.5	2	5
3	1.5	5	10
4	3.0	1	1
5	3.0	2	5
6	3.0	5	10
7	6.0	1	1
8	6.0	2	5
9	6.0	5	10

Development of *M. nivale* primers for the EF-1α gene and sequencing the EF-1α gene for isolates of *M. nivale* var. *majus* and var. *nivale*

PCR products from one isolate of *M. nivale* var. *nivale* (Nr4/N) and one isolate of *M. nivale* var. *majus* (AV9M) amplified using the degenerate EF1/F and EF1/R primers were excised from a low-melting temperature gel (Kramel Biotech, UK) following electrophoresis. Products were recovered using a Wizard® PCR Prep Kit (Promega, Southampton, UK) according to the manufacturer's instructions. Purified DNA was quantified by comparison to Lambda DNA standards of known concentration. Following electrophoresis, unsaturated images were analysed using the Gel Doc system and a standard curve relating image intensity to concentration of Lambda DNA used to calculate the concentration of purified DNA. Purified DNA was then ligated into the pGEM-T vector (Promega) and the plasmid was transformed into competent cells of *Escherichia coli* JM 109 according to the manufacturer's recommendations (Promega). Successful

transformations were confirmed in white colonies by amplification using the M13 F/R primers using the reaction conditions described earlier for diagnostic PCR with program A (Appendix 5) and an anneal temperature of 50°C. Bulk plasmid preparations were prepared by growing positive clones for 16 h in LB broth (Merck). Cultures were purified using the Wizard® Plus SV minipreps DNA purification system (Promega) according to the manufacturer's recommendations. The eluted DNA was diluted in TE buffer and the concentration determined by comparison to lambda DNA standards. Ten µg of eluted DNA were sent to MWG Biotech (Germany) for sequencing using M13 F/R primers.

Sequences were aligned using Clustal W (Thompson *et al.*, 1994) sequence alignment program at www.workbench.sdsc.edu and primers (EFMicF/R) were designed at points conserved between the two sequences to amplify an approximately 840 bp product. These putatively *M. nivale* specific primers were used to amplify DNA from isolates of *M. nivale* var. *majus* and var. *nivale* from several European countries (Appendix 6) (sub-species determined using var. *nivale* and var. *majus* primers described by Nicholson *et al.*, 1996a). DNA was extracted from colonies of *M. nivale* and amplified as described in Chapter 2 (pages 38 and 41), PCR program A was used with an anneal temperature of 50°C. DNA was recovered from PCR products and prepared for sequencing using the method described previously. Eluted DNA (10 µg) was sent to MWG Biotech for sequencing using the *M. nivale* EF-1α gene primers (EFMicF/R).

Restriction site mapping of EF-1α sequences

The sequence consensus obtained for *M. nivale* var. *nivale* and var. *majus* was analysed for restriction enzyme recognition sites which may allow the determination of *M. nivale* sub-species. Sequences were analysed using Webcutter 2.0 at www.firstmarket.com/cutter/cut2.html. Enzymes which digested sequences from each of the two sub-species at different places, were identified as being useful for sub-species discrimination.

The use of the *Sty* I restriction site for *M. nivale* sub-species determination

The restriction enzyme *Sty* I was identified as being of potential use for the identification of *M. nivale* sub-species as this enzyme had three recognition sites on the *M. nivale* var. *nivale* sequence, one of which was also present on the *M. nivale* var. *majus* sequence. A forward primer was designed (EF*Sty*I/F) and matched to the EFMic/R primer, the resulting PCR product incorporated the *Sty* I restriction site for *M. nivale* var. *majus* and two of the restriction sites for *M. nivale* var. *nivale* within the EF-1α gene. Primers EF*Sty*I/F and EFMic/R were used to amplify a 376 bp product from isolates of *M. nivale* var. *nivale* and var. *majus*. Reaction conditions were as described for diagnostic PCR and were adjusted to 50 µl reaction volumes. Following amplification, 30 µl of the PCR reaction was removed to a fresh reaction tube and 3 µl of the *Sty* I restriction enzyme (New England Biolabs, UK) added together with 3 µl of buffer NEB3 (New England Biolabs, UK). Tubes were incubated at 37°C for 1 h after which 15 µl of the reaction mixture was electrophoresed through a 4% agarose gel in order to separate the digested fragments.

Sequence alignment*, M. nivale *var.* nivale *and var.* majus *primer design and testing

Elongation factor 1α Nucleotide sequences were aligned using Clustal W (Thompson *et al.*, 1994) at www.workbench.sdsc.edu and a forward primer designed specifically for each sub-species. A reverse primer (EFMniv/R) which was conserved between the two sub-species was designed. Melting temperature was determined using 'Primers' software at www.williamstone.com. Basic Local Alignment Search Tool (BLAST) was performed using the forward primer sequences and reverse compliment of the reverse primer at www.ncbi.nlm.gov/BLAST/ (Altschul *et al.*, 1997). Primers were tested for cross-reaction against DNA from isolates of pathogens associated with stem-base and seed infections of wheat and also DNA from uninfected host tissue (wheat). DNA was extracted from fungal cultures grown in potato dextrose broth (7 days, 150 rpm, 20°C) (Chapter 2, page 38). DNA was diluted to 1 ng μl^{-1} for use in diagnostic PCR reactions

(Chapter 2, page 41). Confirmation of successful extraction and amplification of fungal DNA was confirmed by amplification using appropriate species specific primer pairs. DNA was extracted from the lower 4 cm of 20 healthy 14 day old wheat (cv. Cadenza) seedlings (GS12) (Chapter 2, page 39) produced from seed in which no *M. nivale* could be detected using the agar plate count method (Chapter 2, page 35). Total DNA was quantified by spectrophotometry and diluted to 40 ng μl^{-1} for use in PCR reactions. Successful extraction of plant DNA was confirmed by amplification using ITS 4 and 5 primers described by White *et al.* (1990). The same PCR program was used for each set of *M. nivale* sub-species specific primers, the program was optimised using a 'Touchdown' method (Don *et al.*, 1991) (Appendix 5). Primers were tested further for cross-reaction between each sub-species (determined using primers described by Nicholson *et al.*, 1996a) to confirm that the *M. nivale* var. *majus* and var. *nivale* primer sites based on the EF-1α gene were conserved in isolates from a range of localities. Microbiological cultures of *M. nivale* or infected grain were obtained from different geographical locations. Cultures of *M. nivale* were isolated from infected grain as described earlier for determination of the incidence of infection in seed lots. Cultures were transferred to a fresh PDA plate, incubated for one week at 20°C before DNA was extracted (Chapter 2, page 38). DNA was amplified using primers previously described for *M. nivale* var. *majus* and var. *nivale* (Nicholson *et al.*, 1996a) and also *M. nivale* var. *majus* and var. *nivale* based on the EF-1α gene. Samples of DNA from *M. nivale* isolates of known sub-species were used as positive controls and PCR reactions containing water were used as negative controls.

Analysis of isolate NRRL 3289

Sub-species determination - morphological and restriction site methods

Attempts were made to determine the sub-species which isolate NRRL 3289 conformed to on the basis of conidia morphology. Cultures were maintained on malt extract agar under NUV lights at 20°C according to the method described by Sanderson *et al.* (1970) and also on PDA under NUV lights at 15°C according to the method of Lees *et al.* (1995). Cultures were examined for sporulation after three weeks. The method of *M. nivale* sub-species determination described by Maurin *et al.* (1995) was also used in an attempt to determine which sub-species isolate NRRL 3289 belonged to. The DNA extraction method described in Chapter 2 (page 38) was used to extract DNA from a fungal culture of isolate NRRL 3289 grown on PDA. DNA was amplified using the universal primers ITS 4 and ITS 5 (White *et al.*, 1990) as described earlier for diagnostic PCR (Chapter 2, page 41) and were adjusted to 50 µl volumes. Two positive controls were used, one containing DNA from an isolate of *M. nivale* var. *nivale* and one containing DNA from an isolate of *M. nivale* var. *majus*. Following the thermocyclic reaction, 30 µl of each PCR reaction was transferred to a fresh reaction tube and 3 µl of the restriction enzyme *Rsa* I (Promega) added together with the restriction enzyme buffer C (Promega). Tubes were incubated at 37°C for 1 h after which 15 µl of each reaction was electrophoresed on a 2% agarose gel (Chapter 2, page 41), 15 µl of the remaining 20 µl of un-digested PCR reaction from one each of the positive controls was also run for size comparison.

Phylogenetic analysis

DNA from the EF-1α gene of isolate NRRL 3289 was sequenced according to the methods described earlier. Primers EFMicF/R were used to amplify the EF-1α gene from DNA extracted from a fungal colony (Chapter 2, page 38). The amplified fragment was

prepared for sequencing as described earlier and sent to MWG Biotech for sequencing. Sequences were aligned with previously obtained sequences for isolates of *M. nivale* var. *nivale* and *M. nivale* var. *majus* using Clustal W (Thompson *et al.*, 1994) multiple sequence alignment program www.workbench.sdsc.edu. Phylogenetic analysis was performed in order to determine the relatedness of the EF-1α sequence for isolate NRRL 3289 towards the previously obtained EF-1α sequences using a distance matrix and rooted dendrogram.

Pathogenicity of isolate NRRL 3289

Pathogenicity tests were performed using isolate NRRL 3289 on wheat leaves according to a method similar to that described by Diamond and Cooke (1999) in order to show that the isolate was pathogenic to wheat and conformed to Koch's postulates (Koch, 1876). Seven wheat leaves (cv. Cadenza) excised from 14-day-old seedlings were placed adaxial side facing upwards in Petri-dishes containing 0.5% water agar amended with 10 mg l^{-1} kinetin (Sigma). Four replicate plates were used. Mycelium from 14-day-old PDA plates of isolate NRRL 3289 was transferred to SDW containing 0.05% Tween 20 (Sigma) by gentle scraping using a sterile glass rod. Fifty µl of the mycelial solution were placed on each leaf, plates were incubated at 22°C for one week. Water containing 0.05% Tween 20 was used as a control and added to the leaves, two isolates of *M. nivale* var. *nivale* (117/1/N) and (139/1/N) and two isolates of *M. nivale* var. *majus* (30/3/M) and (47/2/M) were used for comparison. After one week, infected leaf sections exhibiting visual symptoms of infection were excised from all treatments, surface sterilised using a hypochlorite solution (1% available chlorine) for 3 min and placed on PDA containing 10 mg l^{-1} streptomycin sulphate; plates were incubated at 22°C for one week. DNA was extracted directly from cultures (Chapter 2, page 38) for use in PCR reactions. Attempts were made to determine the sub-species for the culture of *M. nivale* isolate NRRL 3289

produced from leaf isolations using the primers developed in this study, those described by Nicholson *et al.* (1996a) and also the method for sub-species determination described by Maurin *et al.* (1995).

CONSTRUCTION OF INTERNAL STANDARDS

Internal standards (MajIS and NivIS) were constructed for competitive PCR assays based on a method adapted from Förster (1994) from a 1.2kb fragment of the onion (*Allium cepa)* gene alliinase (EMBL accession code L48614). The fragment was amplified using primers ONI/F and ONI/R (Appendix 4) using the same reaction conditions described previously and PCR program A (Appendix 5) with an annealing temperature of 58°C. The 1.2 kb product was excised from the gel following electrophoresis, placed in 1 ml of TE buffer and incubated at 4°C for 16 h. Five µl of gel slice solution was amplified with linker primers (*M. nivale* var. *majus*: EFMaj/LF, EFMniv/LR or *M. nivale* var. *nivale*: EFNiv/LF, EFMniv/LR) with a 'Touchup' PCR program (Appendix 5) consisting of 10 cycles with an annealing temperature of 38°C followed by 20 cycles with an anneal temperature of 50°C. The respective 819 bp linker PCR products were excised from the gel following electrophoresis and placed in 1 ml TE buffer and incubated for 16 h at 4 °C. These linker PCR products consisted of 789 bp of the alliinase gene bordered by the first 10 bp of the var. *majus* or var. *nivale* primer sites. Five µl of gel slice solution was amplified using EFMaj or EFNiv primers and the 'Touchup' program. The resulting PCR products consisted of 789 bp of the alliinase gene bordered by the complete primer sites. PCR products were excised from low-melting temperature gel (Kramel Biotech) following electrophoresis and recovered using a Wizard® PCR Prep Kit (Promega) according to the manufacturer's instructions. Purified DNA was quantified by comparison to Lambda DNA standards and ligated in the pGEM_T vector (Promega). The plasmid was transformed in *Ecsherichia coli* JM 109 according to the manufacturer's recommendations. Successful transformations were confirmed in white colonies by amplification using EFMaj and

EFNiv primers, reaction conditions were as described for diagnostic PCR (Chapter 2 page 41). Bulk plasmid preparations were prepared by growing positive clones for 16 h in LB broth (Merck), cultures were purified using the Wizard® Plus SV minipreps DNA purification system (Promega) according to the manufacturer's recommendations. The eluted DNA was diluted in TE buffer and the concentration determined using spectrophotometry.

PRODUCTION OF STANDARD CURVES

Standard curves were produced for the four sets of primers and internal standards (JBM, JBF, EFMaj and EFNiv). Ten fold dilutions of fungal target DNA were made and amplified with the respective primers. The concentration of DNA two dilutions upwards from that which gave the last visible band was chosen and twelve two-fold dilutions were made from that. Similarly, internal standard DNA (JBMIS, JBFIS, EFMajIS or EFNivIS) was diluted ten-fold, to a concentration two dilutions upwards from that which produced the last visible band and ten two-fold dilutions were made. The middle dilution was chosen and that which was four fold upwards and four fold downward along the dilution series and co-amplified with the dilutions of fungal DNA made previously. The concentration of internal standard DNA which allowed the amplification of fungal DNA over the widest range and with greatest sensitivity was used. Internal standard DNA was prepared in TE buffer in the presence of 10 ng μl^{-1} carrier (Herring sperm) DNA to improve DNA stability during storage, samples were stored at –20°C.

Analysis of PCR products

Amplicons were visualized following gel electrophoresis according to methods described in Chapter 2 (page 41). The ratio in intensity of fungal to internal standard PCR product was used for each set of standards and samples to determine fungal DNA content by relation to the relevant standard curve.

RESULTS

M. NIVALE VAR. *NIVALE* AND VAR. *MAJUS* SPECIFIC PRIMERS

Analysis of rDNA

Sequence alignments of rDNA (Figure 3.1) showed one base pair difference between *M. nivale* var. *nivale* and var. *majus*. Primers were designed to amplify DNA from the two *M. nivale* sub-species (Appendix 4) based on this difference, PCR reactions containing DNA from either sub-species gave a positive PCR signal using either primer set. Increasing the anneal temperature from 54°C to 62°C failed to improve PCR stringency and primers still cross-reacted between sub-species.

Elongation factor-1α gene

Amplification of the EF-1α gene in *M. nivale*

Alignments of EF-1α gene sequences retrieved from sequence databases for several fungal species (Figure 3.2) allowed the design of degenerate primers for the EF-1α gene (Appendix 4). Of the degenerate primers tested, the combination of EF1/F and EF1/R gave fewest spurious amplicons and gave a PCR product which conformed to the expected product size for the EF-1α gene. These primers amplified several smaller products as well as the expected product for the EF-1α gene. As smaller PCR products are preferentially amplified, the yield of the desired product was low.

Optimisation of PCR reaction conditions using Taguchi methods

Adjustment of magnesium, primer and template DNA concentrations showed that 3 µl of $MgCl_2$, 2 µl of each primer and 10 µl of template DNA (treatment 5, Table 3.1) increased yield of the desired product and reduced spurious amplicons during PCR using the degenerate primers. These conditions were adopted in PCR reactions using the

degenerate primers and allowed the amplification of the EF-1α gene from isolates Nr4/N and AV9M of *M. nivale* var. *nivale* and var. *majus* respectively.

***Development of* M. nivale *primers for the EF-1α gene and sequencing the EF-1α gene for isolates of* M. nivale *var.* majus *and var.* nivale.**

Alignments of the EF-1α sequences obtained for *M. nivale* var. *nivale* and var. *majus* using isolates Nr4/N and AV9M, respectively, allowed the design of EF-1α based *M. nivale* primers which in turn allowed the amplification of the EF-1α gene for several isolates of each sub-species from different locations. Elongation factor sequence alignments are given in Figure 3.3.

Restriction site mapping of EF-1α sequences

In order for a particular restriction enzyme to be of use for the identification of *M. nivale* sub-species, the enzyme recognition site must be present at least once for each sub-species but a different number of times between each sub-species. The enzyme must also be commercially available. Enzymes which meet these criteria for the EF-1α gene sequences for *M. nivale* var. *majus* and var. *nivale* and the number of times they occur is given in Table 3.2.

Figure 3.1. rDNA sequence alignments for isolates of *M. nivale* var. *nivale* and *M. nivale* var. *majus* showing *Rsa I* restriction site and primer sequences: ( ) MajITS/*F* ( ) NivITS/F and common reverse , NGMic/R ( ). JBM forward and reverse primers indicated by black underlining.

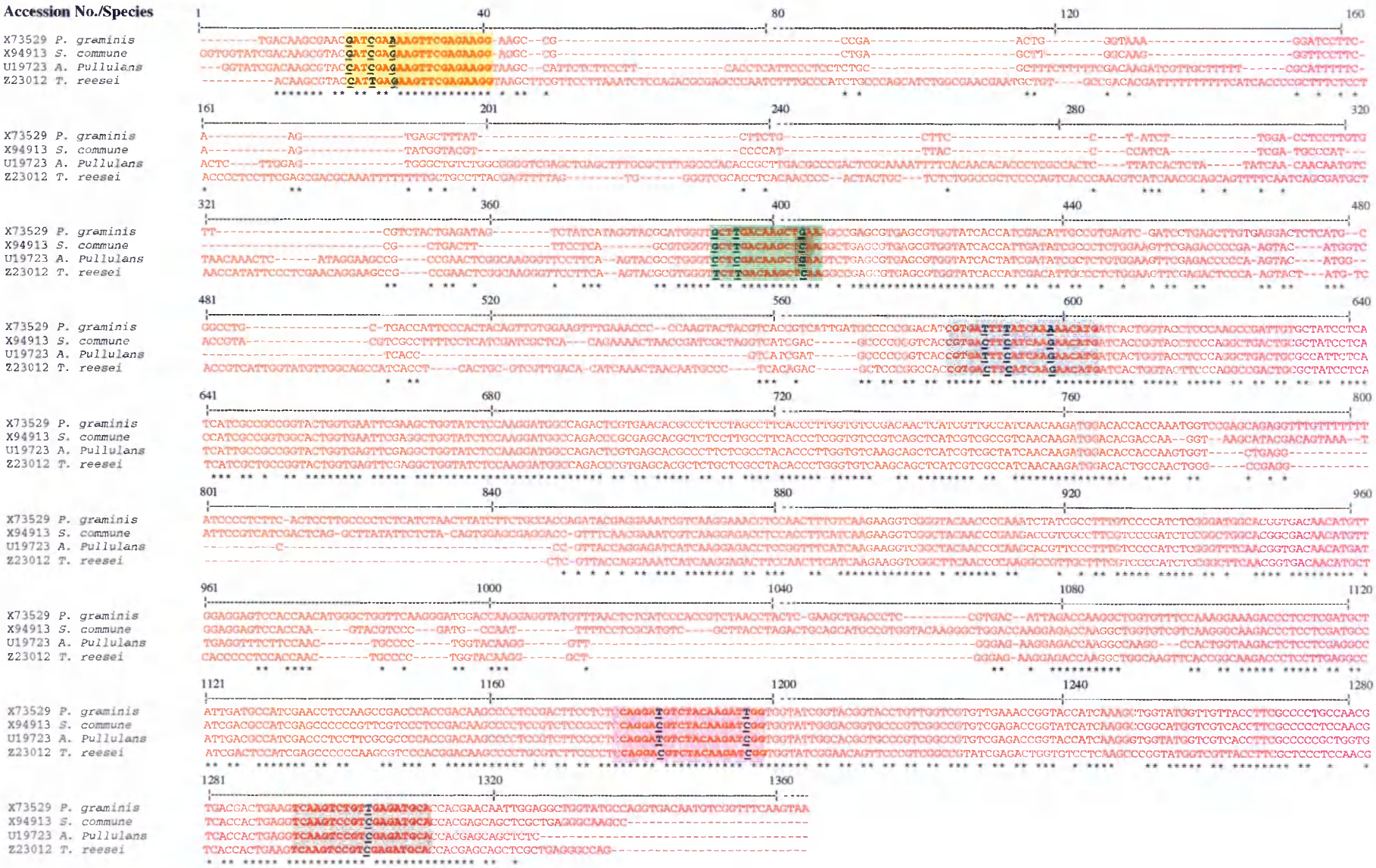


Figure 3.2. Alignment of Partial EF-1α gene sequences for four fungi. * indicates conserved bases. Primer sequences are indicated by shaded areas (□) EF1/F, (□) EF2/F, (□) EF3/F, (□) EF1/R, (□) EF2/R. Base substitutions indicated by black underlining.

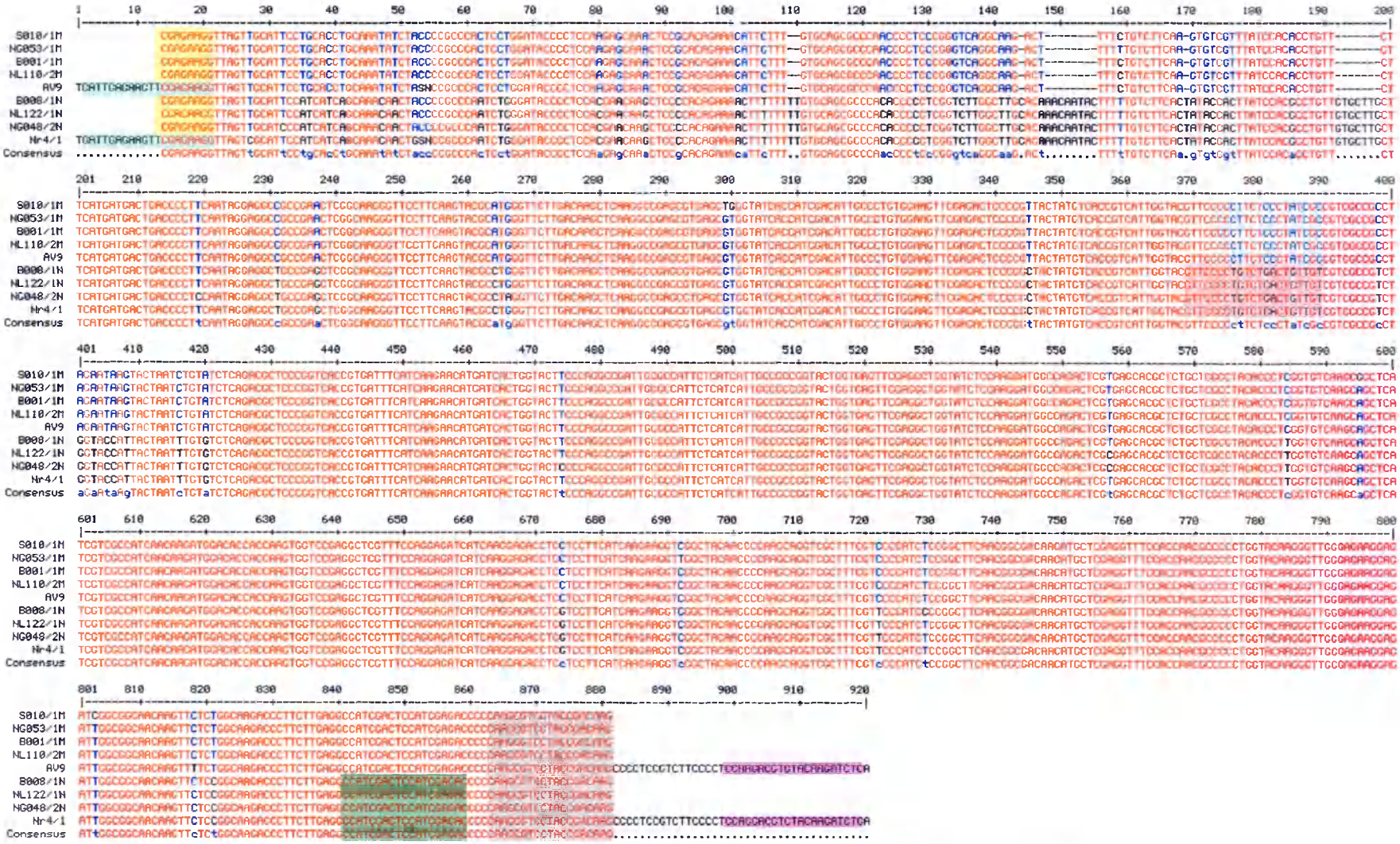


Figure 3.3. Partial EF-1α gene sequences for isolates of *M. nivale* var. *majus* and var. *nivale*. Shaded areas indicate primers sequences used (□) EF1/F, (□) EFMic/F (partial sequence), (□) EFMaj/F, (□) EFniv/R, (□) EFMniv/R, (□) EFMic/R, (□) EF1/R.

Table 3.2. Commercially available restriction enzymes of potential use for the discrimination of *M. nivale* sub-species and the number of times they occur in each sequence.

Enzyme name	No. of cut sites/ sub-species		Enzyme name	No. of cut sites/ sub-species	
	var. *nivale*	var. *majus*		var. *nivale*	var. *majus*
*Aci*I	4	6	*Bso*FI	5	6
*Alu*I	4	2	*Bsp*1286I	2	1
*Alw*26I	4	3	*Bsp*MI	1	2
*Ama*87I	4	2	*Bst*71I	3	2
*Asp*HI	2	1	*Bst*F5I	2	1
*Ava*I	1	2	*Cac*8I	4	2
*Bbv*I	3	2	*Fau*I	2	1
*Bsg*I	1	2	*Fok*I	2	1
*Bsi*HKAI	2	1	*Mbo*II	1	2
*Bsm*AI	4	3	*Nla*IV	2	1
*Bso*BI	1	2	*Sty*I	3	1

The use of the *Sty* I restriction site for *M. nivale* sub-species determination

Restriction enzyme digestion of the EF-1α gene amplified using the EF*Sty*I/F and EFMic/R primers for isolates of *M. nivale* var. *nivale* and var. *majus* resulted in two distinct products for *M. nivale* var. *majus* (55 bp and 321 bp, respectively). Three products were evident for isolates of *M. nivale* var. *nivale* (55 bp, 43bp, and 278 bp, respectively). Despite the use of agarose gels with increased density, discrimination of the 278 bp and 321 bp products was extremely difficult. Although the technique would allow the determination of the *M. nivale* sub-species present in a sample when only one was present, it proved to have no advantage over the use of sub-species specific primers and would also be more expensive if implemented.

Sequence alignment, *M. nivale* var. *nivale* and var. *majus* primer design and testing

The EF-1α sequence alignment for *M. nivale* var. *nivale* and var. *majus* showed several base differences conserved to each sub-species (Figure 3.3). A forward primer was designed for each sub-species whereas a common reverse primer was used. Comparison of the primer sequences using BLAST analysis indicated that the sequences were not present in wheat or fungal species associated with wheat.

The *M. nivale* var. *nivale* and *M. nivale* var. *majus* primers showed no cross-reaction between the two sub-species or towards several fungal pathogens which can cause Fusarium diseases of wheat or can be present in the stem-base complex of wheat (Table 3.3). The primers also showed no cross-reaction towards plant DNA from the uninfected host species (*Triticum aestivum* L.)

All isolates of *M. nivale* tested from Europe, New Zealand and Japan gave a positive PCR signal using either the *M. nivale* var. *nivale* or the *M. nivale* var. *majus* primers developed in this study (Appendix 6). The same isolates also gave a positive PCR signal using the corresponding *M. nivale* sub-species primers described by Nicholson *et al.* (1996a). Thus both sets of primers were in agreement for these isolates. The primers were also in agreement for four of the five isolates of *M. nivale* from North America. One isolate from Canada (NRRL 3289) gave a positive *M. nivale* var. *nivale* PCR signal with the primers developed in this study however, no signal was obtained using either of the primer sets described by Nicholson *et al.* (1996a).

Table 3.3. Code and origin of fungal isolates.

Species	Code	Origin	PCR Signal (+/-)
F. avenaceum	Fa 131	UK	-
F. avenaceum	Fa 721	Germany	-
F. avenaceum	Fa 303	France	-
F. culmorum	Fc 121/1	Unknown	-
F. culmorum	Fu 5	UK	-
F. culmorum	Fc K	Unknown	-
F. culmorum	Fc 95w	Unknown	-
F. culmorum	Fu 42	UK	-
F. culmorum	Fc 36	Unknown	-
F. culmorum	Fc J	Unknown	-
F. graminearum	Fg 507	France	-
F. graminearum	Fg 86	France	-
F. graminearum	Fg 131	UK	-
F. poae	Fu 53P	Norfolk, UK	-
F. poae	F733	France	-
F. poae	Fp 113/1	Unknown	-
F. poae	Fp 4/4343/2	UK	-
Tapesia yallundae	194(17.38)	UK	-
Tapesia yallundae	HATy1	Unknown	-
Tapesia acuformis	132(72)	UK	-
Tapesia acuformis	HATa1	Unknown	-
Rhizoctonia cerealis	Rc 88/303	Saffron Walden, UK	-
Rhizoctonia cerealis	Rc 84/280	Cambridge, UK	-
Triticum aestivum L.	N/A	N/A	-

- no PCR signal detected, using EF-1α gene *M. nivale* var. *nivale* or *M. nivale* var. *majus* primers.

N/A Not Applicable

Analysis of isolate NRRL 3289

Sub-species determination - morphological and restriction site methods

The growth of cultures of NRRL 3289 under the conditions described by Sanderson *et al.* (1970) or Lees *et al.* (1995) failed to induce sporulation of the isolate and therefore conidial morphology could not be used for sub-species determination for NRRL 3289. The method for *M. nivale* sub-species determination, amplification of rDNA using primers described by White *et al.* (1990) followed by restriction enzyme digestion using *Rsa* I, showed that this isolate lacked the *Rsa* I restriction site and thus did not conform to *M. nivale* var. *majus*.

Phylogenetic analysis

The EF-1α sequence for isolate NRRL 3289 had a mean sequence distance (number of base differences/ total number of bases) score of 0.00725 towards the EF-1α sequences for isolates of *M. nivale* var. *nivale* and 0.0706 towards EF-1α sequences for *M. nivale* var. *majus* (Table 3.4). The mean sequence distance score between the EF-1α sequences previously obtained for the four isolates of *M. nivale* var. *nivale* was 0.0042 and was 0.003 between the five isolates of *M. nivale* var. *majus*. The mean sequence distance between the four original isolates of *M. nivale* var. *nivale* and the five isolates of *M. nivale* var. *majus* was 0.0719. It was, therefore, apparent that isolate NRRL 3289 belongs to *M. nivale* var. *nivale* (Figure 3.4). The EF-1α sequence for NRRL 3289 showed 100 % homology towards both the forward primer site designed for *M. nivale* var. *nivale* and the reverse primer site which was conserved between the two sub-species.

Table 3.4. Distance matrix for isolates of *M. nivale* var. *nivale* and var. *majus*.

Isolate code/ Number	Distance between sequences (No. of base differences/ total No. of bases) (x 10^{-1})									
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
NL110/2M (1)	0.00	0.00	0.05	0.01	0.02	0.69	0.74	0.69	0.69	0.69
B001/1M (2)	0.00	0.00	0.05	0.01	0.02	0.69	0.74	0.69	0.69	0.69
S010/1M (3)	0.05	0.05	0.00	0.06	0.07	0.74	0.79	0.74	0.74	0.74
AV9M (4)	0.01	0.01	0.06	0.00	0.04	0.71	0.75	0.71	0.71	0.71
NG053/1M (5)	0.02	0.02	0.07	0.04	0	0.70	0.75	0.70	0.71	0.70
NL122/1N (6)	0.69	0.69	0.74	0.71	0.7	0.00	0.05	0.02	0.02	0.06
NG048/2N (7)	0.74	0.74	0.79	0.75	0.75	0.05	0.00	0.07	0.07	0.11
B008/1N (8)	0.69	0.69	0.74	0.71	0.7	0.02	0.07	0.00	0.02	0.06
Nr4/N (9)	0.69	0.69	0.74	0.71	0.71	0.02	0.07	0.02	0.00	0.06
NRRL3289(10)	0.69	0.69	0.74	0.71	0.7	0.06	0.11	0.06	0.06	0.00

- Comparisons between isolates *M. nivale* var. *nivale* and var. *majus* (Mean = 0.72 x 10^{-1})
- Comparisons between isolates of *M. nivale* var. *majus* (Mean = 0.03 x 10^{-1})
- Comparisons between isolates of *M. nivale* var. *nivale* (Mean = 0.04 x 10^{-2})
- Comparison between isolate NRRL 3289 and *M. nivale* var. *majus* isolates (Mean = 0.71 x 10^{-1})
- Comparison between isolate NRRL 3289 and *M. nivale* var. *nivale* isolates (Mean = 0.07 x 10^{-1})

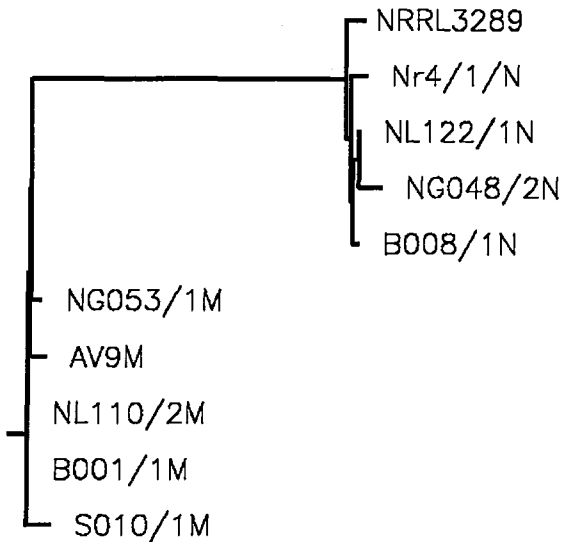


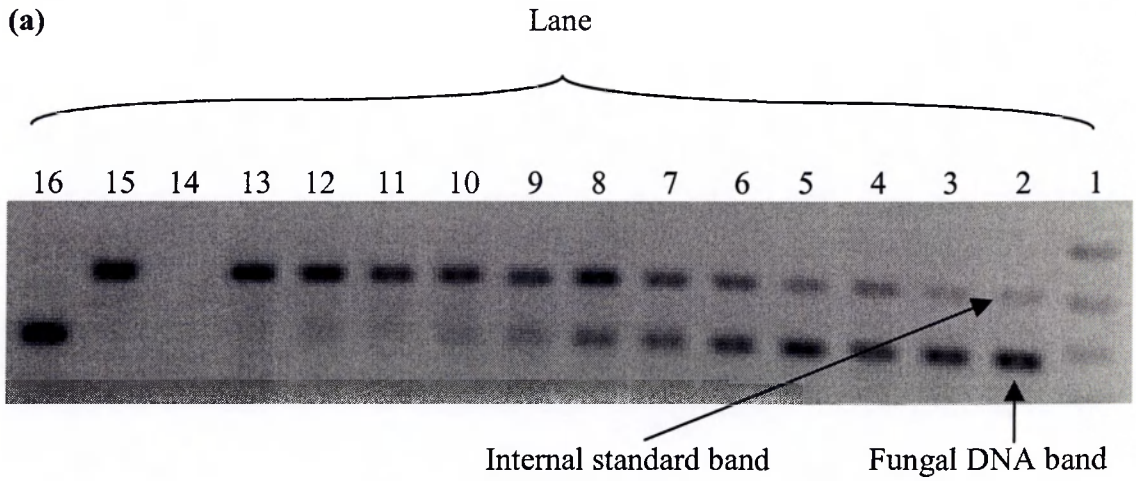
Figure 3.4. Rooted dendrogram for EF-1alpha sequences for isolates of *M. nivale* var. *nivale* and var. *majus*.

Pathogenicity of isolate NRRL 3289

Severe visual symptoms of disease were apparent on all wheat inoculated with fungal mycelium with chlorotic lesions clearly visible. No symptoms of infection were present on the water control. Symptoms caused by NRRL 3289 did not differ in incidence or severity from those caused by the isolates of *M. nivale* var. *nivale* and var. *majus*. Colonies of *M. nivale* were re-isolated from all infected leaves included those inoculated with mycelium of NRRL 3289. No colonies were isolated from the water controls. DNA extracted from the re-isolated colonies of isolate NRRL 3289 was successfully amplified by the EF-1α primers for *M. nivale* var. *nivale* developed in this study and was not amplified by EF-1α primers for *M. nivale* var. *majus*. Isolate NRRL 3289 therefore was pathogenic and did conform to Koch's postulates. DNA from re-isolated colonies of NRRL 3289 could not be amplified using the *M. nivale* var. *nivale* or var. *majus* primers described by Nicholson *et al.* (1996a) and could be amplified using primers ITS 4 and ITS 5 though was not digested by the restriction enzyme *Rsa* I.

CONSTRUCTION OF INTERNAL STANDARDS AND PRODUCTION OF STANDARD CURVES

Internal standards were successfully produced for all four primer sets; JBM, JBF, EFNiv and EFMaj (internal standards for JBF and JBM were produced by S.G. Edwards). Amplification of internal standard DNA using the respective primers resulted in products of expected size (628, 534, 831, and 827 bp, respectively). Standard curves were produced for each internal standard and respective fungal DNA combination (Figures 3.5 to 3.8). The limits and range of detection, and concentration of internal standard used is given in Table 3.5. *Microdochium nivale* competitor (MicIS) DNA (2.9 fg) or *Fusarium* spp. competitor (FusIS) DNA (38.3 fg) (determined previously by S.G. Edwards) was added in 10 µl to quantitative reactions and 10 µl of sample DNA was added. *Microdochium nivale* DNA was diluted two fold over the range 1.6×10^{-2} ng to 3.1×10^{-5} ng. *Fusarium culmorum* DNA was diluted two fold over the range 6.4×10^{-2} ng to 7.9×10^{-6} ng. Ten µl of each dilution was added to separate quantitative reactions to produce a standard curve. *Microdochium nivale* var. *nivale* competitor (EFNivIS) DNA (89.7 fg) or *Microdochium nivale* var. *majus* competitor (EFMajIS) DNA (67.4 fg) was added in 10 µl to quantitative reactions and 10 µl of sample DNA was added. *Microdochium nivale* var. *nivale* DNA was diluted two fold over the range 10.8 ng to 0.0053 ng *M. nivale* var. *majus* DNA was diluted two fold over the range 4.3 ng to 0.0021 ng. All constituents outlined for diagnostic PCR's were adjusted for quantitative PCR to a final a reaction volume of 50 µl. The quantitative assay developed for *M. nivale* was used to quantify the fungal standards used for the standard curves for each sub-species.



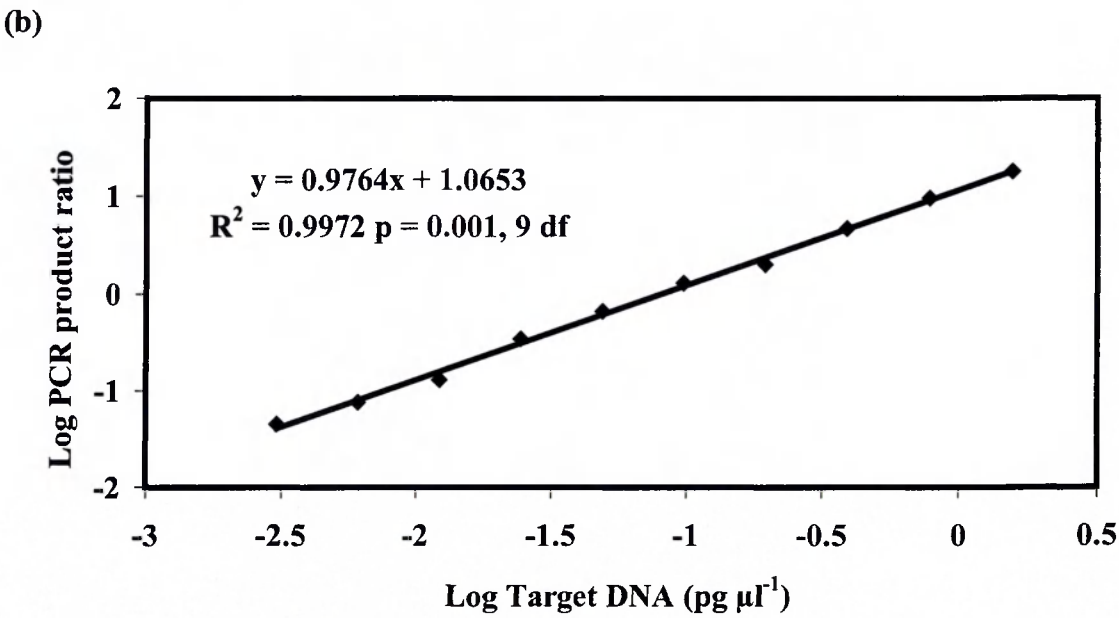


Figure 3.5. Standard curve produced by the co-amplification of *M. nivale* DNA (along a dilution series) and Internal standard DNA (at a constant amount) using JBM primers (a) Fungal and internal standard PCR products; Lane 1 = ϕX174/*Hinc*I size marker, lanes 2-13 = two fold dilutions of fungal target DNA from 1.6 to 0.003 pg μl^{-1}, lane 14 = negative control, 15 = positive internal standard DNA, 16 = positive fungal DNA. (b) Relationship between fungal DNA and PCR product ratio DNA.

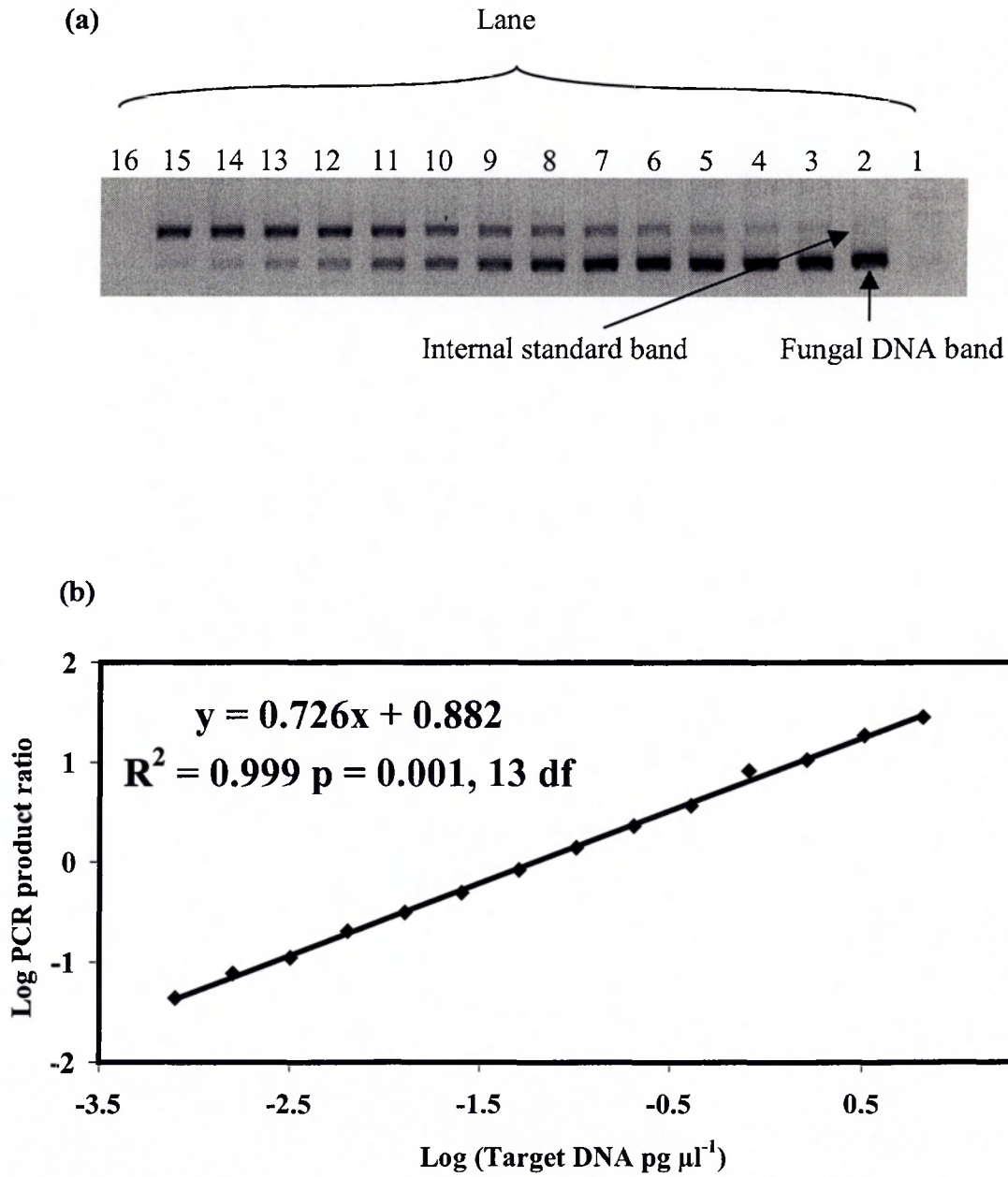


Figure 3.6. Standard curve produced by the co-amplification of *Fusarium* spp. DNA (along a dilution series) and Internal standard DNA (at a constant amount) using JBF primers (a) Fungal and internal standard PCR products; Lane 1 = ϕX174/*Hinc*I size marker, lanes 2-15 = two fold dilutions of fungal target DNA from 6.4 pg μl^{-1} – 0.0008 pg μl^{-1}, lane 16 negative control. (b) relationship between fungal DNA and PCR product ratio DNA.

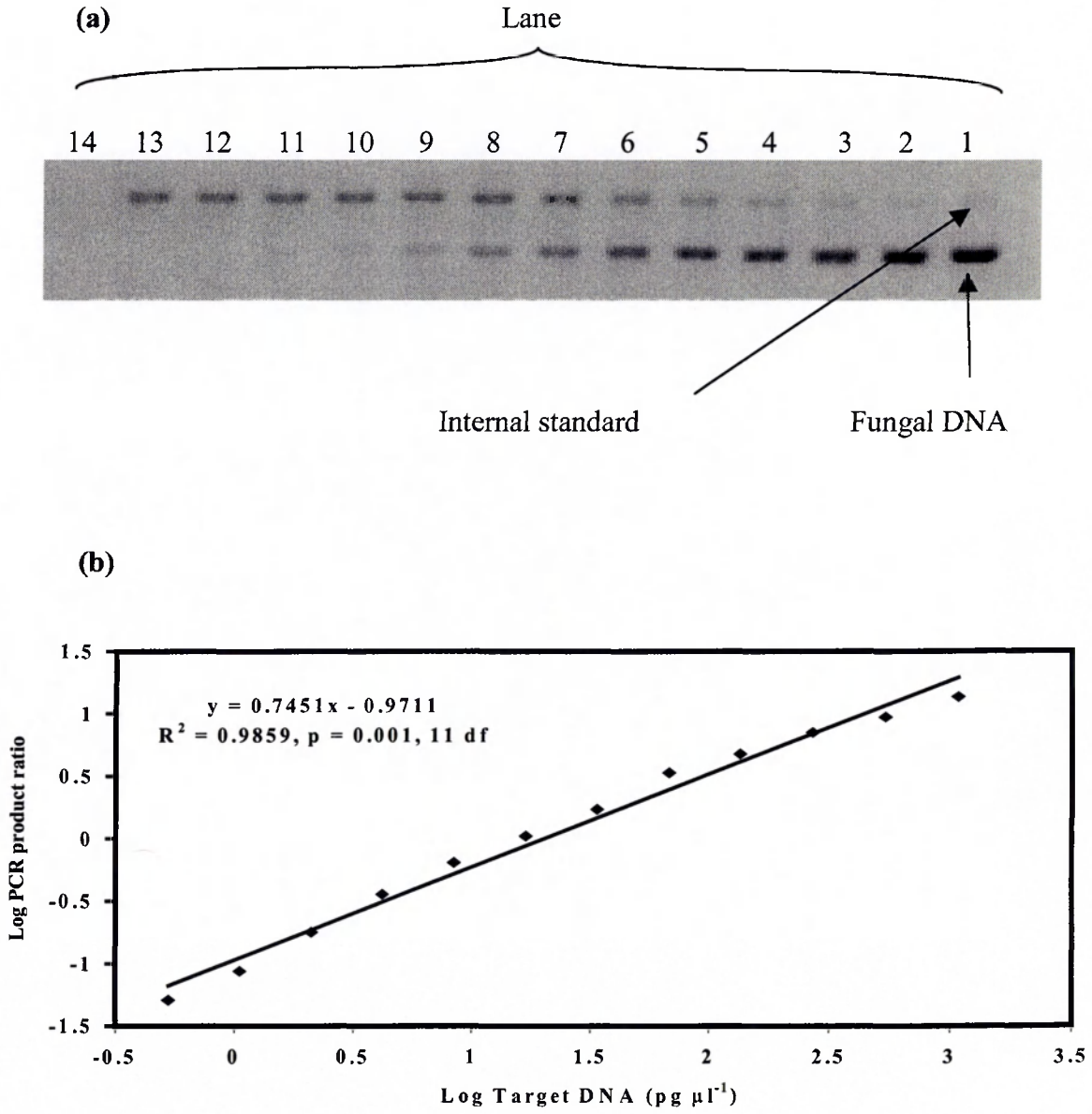
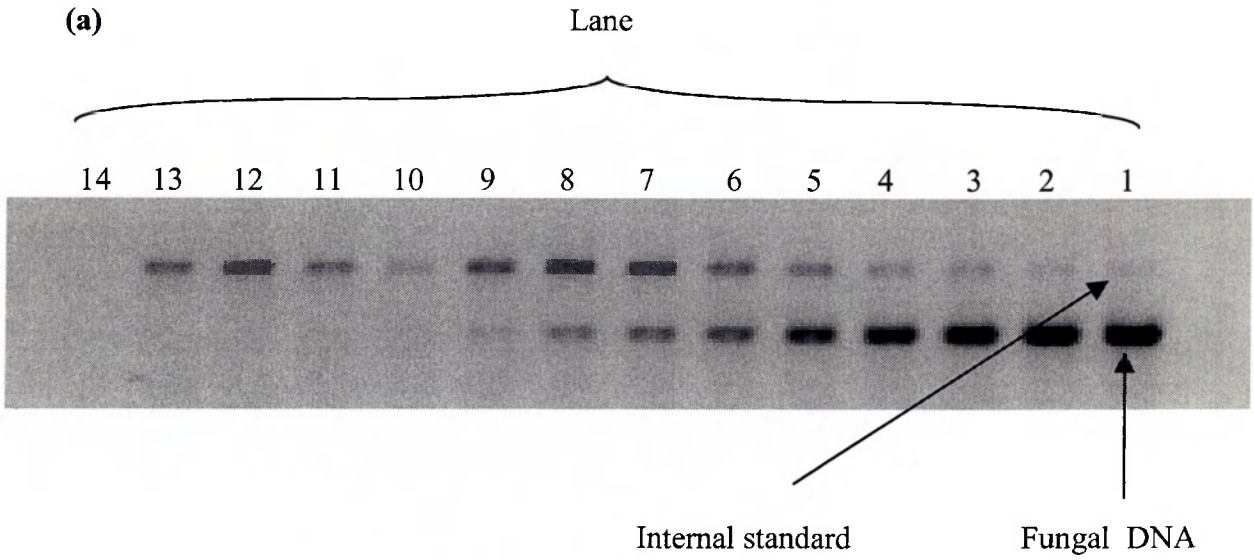


Figure 3.7. Standard curve produced by the co-amplification of *M. nivale* var. *nivale* DNA (along a dilution series) and Internal standard DNA (at a constant amount) using EFNiv primers (a) Fungal and internal standard PCR products; Lanes 1-12 = two fold dilutions of fungal target DNA from 1080 pg μl^{-1} - 0.53 pg μl^{-1}, lane 13 positive internal standard control, 14 negative control. (b) relationship between fungal DNA and PCR product ratio DNA.



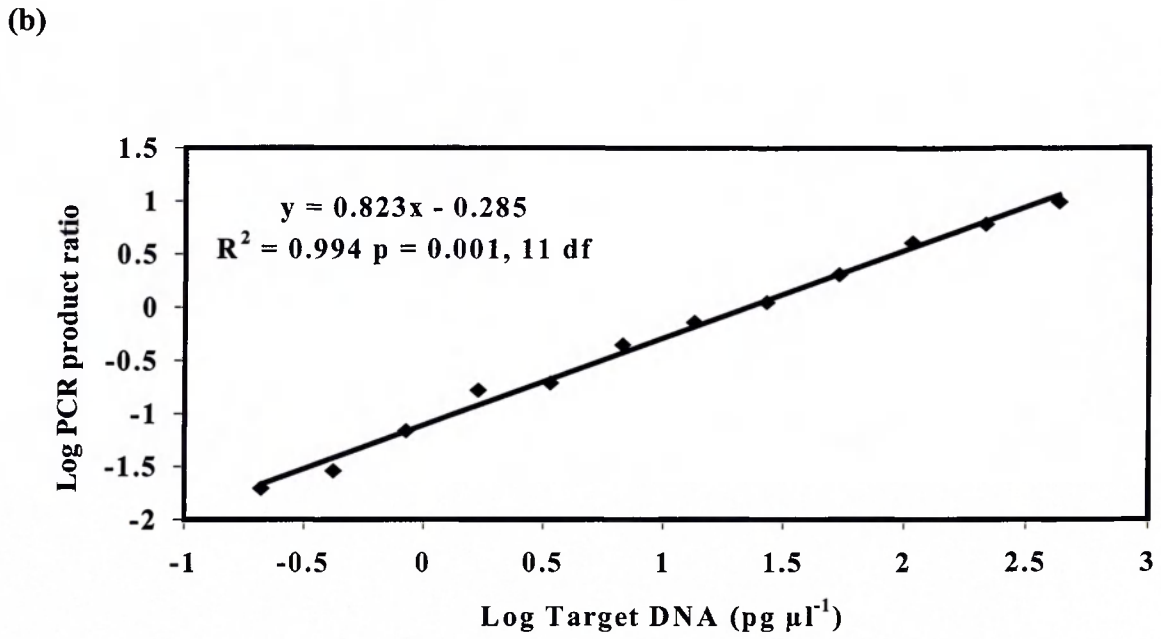


Figure 3.8. Standard curve produced by the co-amplification of *M. nivale* var. *majus* DNA (along a dilution series) and Internal standard DNA (at a constant amount) using EFMaj primers (a) Fungal and internal standard PCR products; Lanes 1-12 = two fold dilutions of fungal target DNA from 430 pg μl^{-1}– 0.21 pg μl^{-1}, lane 14 positive internal standard control, 15 negative control. (b) relationship between fungal DNA and PCR product ratio DNA.

Table 3.5. Details of quantitative PCR assays for JBM, JBF, EFMaj and EFNiv primers and internal standards.

Primers	Species detected	Upper limit of detection	Lower limit of detection	Conc. of internal standard
JBM	*M. nivale*	1.62×10^{1} pg μl^{-1}	3.12×10^{-3} pg μl^{-1}	2.92 fg
JBF	*Fusarium* spp.	6.40 pg μl^{-1}	7.93×10^{-4} pg μl^{-1}	38.37 fg
EFMaj	*M. nivale* var. *majus*	4.34×10^{2}pg μl^{-1}	0.21 pg μl^{-1}	67.43 fg
EFNiv	*M. nivale* var. *nivale*	1.08×10^{3} pg μl^{-1}	0.53 pg μl^{-1}	89.71 fg

DISCUSSION

The usefulness of any genomic region for the development of a PCR based diagnostic test is dependent upon its heterogeneity compared to other species of fungi which occur within the same niche (as well as the host plant species). The homology of sequence identity within isolates of the chosen fungal species is also an important requirement. Where large taxonomic differences between the test species of fungi and other related species exist, the region of choice for both diagnostic and phylogenetic studies appears to be the ITS region due to its high copy number and availability of sequence information. However, where large genomic differences are not evident, it is necessary to use active gene sequences for such studies. Carbone and Kohn (1999) stated that the suite of nuclear ribosomal DNA sequences accessioned in databases was not sufficient, particularly at the intraspecific level. The authors described what they termed universal primers for fungal protein encoding genomic regions based exclusively on nucleotide sequences found in databases. These putatively universal primers were based

on genomic regions encoding the intergenic spacer (IGS) region of the nuclear ribosomal DNA repeat, portions of the translation elongation factor 1 alpha (EF 1-α), calmodulin and chitin synthase 1 genes as well as genes encoding actin and ras protein. The publication of the primers by Carbone and Kohn (1999) came after the initial work in the present study.

The ITS region of rDNA was chosen in this study as it is a high copy number region and primers based on the ITS have been reported for other fungal pathogens of wheat (Beck and Ligon, 1995, Beck *et al.*, 1996). Initial studies revealed little heterogeneity between *M. nivale* var. *majus* and var. *nivale* within the ITS and only one base pair difference at the *Rsa* I restriction site. Primers designed on this difference cross-reacted between the two sub-species despite attempts to optimise the stringency of the amplification by adjustment of the anneal temperature. Maurin *et al.* (1995) reported that the *Rsa I* restriction site present in the ITS region of rDNA of *M. nivale* was conserved among European isolates conforming to *M. nivale* var. *majus*. Isolates of *M. nivale* which did not exhibit the *Rsa I* site were termed *M. nivale* var. *nivale*. Mahuku *et al.* (1998) used the presence or absence of the *Rsa I* site to distinguish the sub-species of turfgrass isolates of *M. nivale*. All of 100 isolates tested lacked the *Rsa I* restriction site and were termed *M. nivale* var. *nivale*. They used the universal primers described by White *et al.* (1990) to amplify rDNA. However, isolates which were not digested by *Rsa 1* may not have been *M. nivale*, the enzyme may have been inhibited or they may have been isolates of the genetically diverse *nivale* variety which lacked this feature.

Primers specific to *M. nivale* were designed and tested by Syngenta based on the rDNA sequences. Primers were also obtained from Syngenta which were designed at points conserved within rDNA sequences from the major species of *Fusarium* which cause ear blight of wheat (*F. avenaceum, F. culmorum, F. graminearum* and *F. poae*). Both sets of primers targeted the ITS region of rDNA in these pathogens. These primers would not allow differentiation between individual sub-species and species respectively but could prove useful for the detection of pathogen inoculum from the two principle taxonomic

groups which are responsible for Fusarium diseases of wheat. Seedling infections caused by *M. nivale* and *Fusarium* spp. favour different optimal environmental conditions (Colhoun *et al.*, 1968, Millar and Colhoun, 1969) and have also been shown to have differing responses to fungicides (Pettitt *et al.* 1993, Jennings *et al.*, 2000).

The set of coding and non-coding regions of rDNA have been used to identify variability and homology at various taxonomic levels due to their differential rates of evolution (White *et al.* 1990). White *et al.* (1990) reported areas of rDNA which were highly conserved between many species of filamentous fungi. These primer sites have proved useful for taxonomic studies within *Fusarium* (Bateman *et al.*, 1996, Waalwijk *et al.*, 1996, Lee *et al.*, 2000). Waalwijk *et al.* (1996) and O'Donnell and Cigelnik (1997) reported the co-existence of two ITS2 sequences within strains of *Fusarium* belonging to species within the *Giberella fujikuroi* complex which were termed type I and type II. The same authors reported low divergence between the same species within ITS1. The forward primer for *Fusarium* spp. used in this study is based within the low divergent ITS1 region and is designed at points which are conserved between the four *Fusarium* species tested (*F. avenaceum, F. culmorum, F. graminearum* and *F. poae*). These are the major species of *Fusarium* responsible for *Fusarium* diseases of cereals in the UK. The reverse primer shows 94.4% and 100% homology respectively towards the type I and type II ITS2 species described by O'Donnell and Cigelnik (1997) with a single cytosine base insertion 13 bp from the 3' end of the reverse primer site the only difference. As the specificity of a primer is determined mainly by the 3' end (Newton and Graham, 1997), a single base mismatch in the middle of the primer would be unlikely to affect the specificity or efficiency of amplification. Thus, despite the co-existence of two ITS sequences within some species of *Fusarium*, the primers developed by Syngenta and used in this study are sufficiently conserved to detect both types and the reverse primer described may be useful in the detection of the *G. fujikuroi* complex. No separate ITS types have been reported in the species of *Fusarium* which the primers used in this study were based on.

Little polymorphism was evident between the two *M. nivale* sub-species within rDNA. Consequently, the gene encoding the elongation factor 1-α (EF 1-α) protein was identified as a region for primer design as variability within the EF 1-α gene for species of *Fusarium* were reported by O'Donnell *et al.* (1998). The EF 1-α is ubiquitous and one of the most abundant proteins in eukaryotes making up to 2% of total protein in growing cells (Slobin, 1980). It is an essential component in protein synthesis in eukaryotes, carrying aminoacyl-tRNA to the A site of ribosome-mRNA-peptidyl-tRNA complex. Unlike the ITS region, no universal primers (White *et al.*, 1990) were available for the EF 1-α gene when the work was performed. Therefore, degenerate primers were developed from alignments of EF 1-α gene sequences for several species of fungi retrieved from molecular databases. Wendland and Kothe (1997) were able to isolate the EF 1-α gene from the fungus *Schizophyllum commune* using degenerate primers derived from sequence alignments of fungal EF 1-α sequences. O'Donnell *et al.* (1998) used degenerate primers based on EF 1-α gene sequences of *Trichoderma reesei* and *Histoplasma capsulatum* to amplify partial sections of the EF 1-α gene from isolates of *F. oxysporum* for sequencing. Aoki and O'Donnell (1999) developed species specific primers based on the EF 1-α gene of *Fusarium pseudograminearum.* Their primers amplified DNA from 17 isolates of the fungus from USA and Africa and did not amplify DNA from other closely related species of *Fusarium* (*F. graminearum, F. cerealis, F. culmorum, F. sambucinum* and *F. venenatum*) from North America, Europe, Asia, South America or Africa. Nicholson *et al.* (1996a) who developed primers for the two sub-species of *M. nivale* showed that their primers did not cross-react between sub-species or amplify DNA from common stem-base pathogens of wheat (*F. culmorum, F. avenaceum, F. poae, F. graminearum, T. yallundae, T. acuformis* and *R. cerealis*). They used 12 isolates of *M. nivale* var. *nivale* all of which were from either Shropshire UK, Berkshire UK or of unknown origin and 12 isolates of *M. nivale* var. *majus* all of which were from the UK. The primers developed in this study

gave positive PCR signals for DNA from 44 isolates of *M. nivale* var. *majus* and 38 isolates of *M. nivale* var. *nivale* from Europe, Japan, New Zealand and North America resulting in single PCR products of expected size and no cross-reaction between sub-species. The *M. nivale* sub-species determined by the EF 1-α based primers was confirmed by the primers developed by Nicholson *et al.* (1996a) in all cases with the exception of one isolate (NRRL 3289) from Canada. The EF 1-α based primers gave a positive PCR signal for *M. nivale* var. *nivale* for this isolate whereas no PCR signal was obtained with the primers described by Nicholson *et al.* (1996a). This isolate also lacked the *Rsa I* restriction site in rDNA amplified using primers described by White *et al.* (1990) and therefore did not conform to *M. nivale* var. *majus* described by Maurin *et al.* (1995). Sequence analysis of the EF 1-α gene showed that NRRL 3289 had a greater relatedness to *M. nivale* var. *nivale* than *M. nivale* var. *majus* and also confirmed that the primer sites were present within the EF 1-α gene.

Isolate NRRL 3289 was obtained from the United States Department of Agriculture culture collection. The isolate was originally deposited by W. L. Gordon in 1967 as strain 3125 (O'Donnell, pers. comm.). Logrieco *et al.* (1991) examined the taxonomic disposition of isolate NRRL 3289. They compared it to two other isolates of *M. nivale* by examination of the D2 region of rRNA and comparison to morphological characteristics. All three isolates had the same rRNA sequence and exhibited macroscopic and microscopic features of *M. nivale.* As the two isolates of *M. nivale* used for comparison were identified as *M. nivale* var. *majus* by morphological characteristics, isolate NRRL 3289 was also classified as being *M. nivale* var. *majus.* In the present study, isolate NRRL 3289 has been identified as being *M. nivale* var. *nivale.* The contradiction to the findings of Logreico *et al.* (1991) is likely to be a result of the absence of an *M. nivale* var. *nivale* isolate in their study. Had var. *nivale* isolates been examined by Logreico *et al.* (1991) it is likely that they would have found no difference in the D2 rRNA sequences as was the case

for the 5.8S gene in this study. This would therefore preclude polymorphisms within ribosomal genes as taxonomic criteria for the division of sub-species within *M. nivale*. The authors do not state why they did not characterise isolate NRRL 3289 on the basis of fungal structure morphology though this may be a result of sporulation by this isolate being what they term as sparse. The results of Logrieco *et al.* (1991) add further confirmation that isolate NRRL 3289 is indeed *M. nivale*.

Isolate NRRL 3289 also conformed to Koch's postulates in this study proving pathogenic towards wheat leaves. Diamond and Cooke (1999) reported that the pathogenicity of isolates of *M. nivale* towards sections of excised wheat leaves was correlated with the pathogenicity of the same isolates towards wheat ears. Although the majority of isolates tested in this study (99%) could be detected with the primers described by Nicholson *et al.* (1996a) and those in this study, the presence of isolates such as NRRL 3289 in wild type populations could lead to false negative detections and/or underestimation of *M. nivale* var. *nivale* inoculum in infected plant material. The EF 1-α var. *majus* and var. *nivale* primers also detected pathogen inoculum in infected seed and seedlings and did not amplify DNA from the host (wheat).

Internal standards were constructed for the four primer sets according a method adapted from that described by Förster (1994) who used a single linker primer to produce a homologous internal standard. The use of two linker primers to amplify (foreign) onion DNA produced heterologous internal standards and combined with the use of the 'Touchup' PCR program reduced the formation of heteroduplexes and spurious amplicons, respectively. False negatives in PCR reactions can often be the result of inhibition caused by inhibitors extracted along with the template DNA (Wilson, 1997). The use of an internal standard in PCR reactions involving template DNA from plant samples was cited by Judelson and Tooley (2000) as being a way of avoiding false negatives caused by inhibitors.

Standard assay curves were developed which allowed the estimation of pathogen DNA in samples. The quantitative assays for *M. nivale* and *Fusarium* using primers developed by Syngenta showed improved sensitivity compared to those previously available for the individual sub-species of *M. nivale* (Nicholson *et al.*, 1996a) and species of *Fusarium* (Nicholson *et al.*, 1998). This is as a result of these primers being based on the highly repeated ITS region of rDNA. The quantitative assays developed for the two sub-species of *M. nivale* in this study however, did not show significantly improved sensitivity compared to those described by Nicholson *et al.* (1996a). This may be a result of the EF 1-α gene being of similar copy number to the genomic regions which the primers designed by Nicholson *et al.* (1996a) are based on. However, the quantitative assays developed in this study allow improved comparison between each *M. nivale* sub-species as they are based on the same genomic region in each pathogen. Also, the concentration of fungal standards used for both standard assay curves was determined using the quantitative assay previously described for *M. nivale*. Höxter *et al.* (1991) were able to quantify *M. nivale* inoculum *in planta* using an indirect ELISA and reported the limit of sensitivity of the assay to be 0.1 pg mycelium μl^{-1}. The antibodies raised reacted to six isolates of *M. nivale* from different geographic locations and showed no cross-reaction to other species of *Fusarium* however, the assay did not differentiate between the *M. nivale* sub-species.

Simpson *et al.* (2000) used the quantitative assays for *M. nivale* var. *nivale* and var. *majus* previously developed by Nicholson *et al.* (1996a) in an investigation of pathogenicity of the two sub-species towards different host species. They used mycelial suspensions from the two sub-species at different rates. However, quantification of the separate sub-species in each mixture using the previously described assays resulted in a disparity to that expected of almost ten-fold in some cases. This may be a result of the primers for each pathogen being based on genomic regions with different copy numbers.

Judelson and Tooley (2000) developed a highly sensitive quantitative assay for the detection of the oomycete *Phytophthora infestans in planta.* They developed primers

which targeted a highly repeated family of nuclear DNA specific to the species and reported that the assay was about 100 times more sensitive than previously described assays based on the ITS region of rDNA. An alternative approach to developing quantitative tests for the two sub-species of *M. nivale* with improved sensitivity may therefore be to design primers based on highly repeated regions of nuclear DNA. He *et al.* (1994) were able to improve the senitivity of primer pairs for the detection of species of mycobacteria by between 100 and 1000 fold by altering the positions within the target sequence that the primers were based on. In the present study, the points at which the sub-species specific primers could be located was restricted to the points within the EF 1-α gene which differed between the two pathogens. Optimisation of the forward primer melting temperatures to match the reverse primer was achieved by altering the length of the primers. This had the added advantage of allowing the same PCR programme to be used for the detection of both sub-species simultaneously.

Phylogenetic analysis of the EF 1-α gene sequences revealed considerable homogeneity between all isolates although the two sub-species could clearly be distinguished. Isolates conforming to *M. nivale* var. *majus* showed slightly more homogeneity than isolates of *M. nivale* var. *nivale.* Although the sample size used in this study is relatively small, the geographic locations covered is relatively large within Northern Europe. The conclusions based on this small sample size are in keeping with those of Lees *et al.* (1995) who recorded greater diversity within *M. nivale* var. *nivale* than var. *majus.* The authors suggested that this diversity maybe a result of the ability of var. *nivale* to under go heterothallic reproduction under natural conditions, a feature that may not be present in var. *majus.*

CHAPTER 4

APPLICATION OF MOLECULAR DIAGNOSTIC ASSAYS FOR THE DETERMINATION OF *M. NIVALE* CONTAMINATION OF WHEAT SEED AND THE RELATIONSHIP WITH SEEDLING BLIGHT DISEASE

INTRODUCTION

Seed-borne *M. nivale* is the primary source of inoculum for Fusarium seedling blight in the UK (Colhoun, 1970) and causes disease when contaminated seed is sown and environmental conditions favour disease development. The approach traditionally employed to determine the severity of *M. nivale* contamination of seed batches is to estimate the percentage of seeds that are contaminated by *M. nivale* based on a representative sub-sample of the seed batch in question. Seed is incubated in paper towels, sand or compost and the number of the resulting seedlings which exhibit disease symptoms is recorded. Symptoms of seedling blight can be caused by several species of *Fusarium* (Colhoun, 1970) as well as *M. nivale* and are influenced profoundly by environmental conditions. Therefore, estimations of the degree of *M. nivale* contamination of seed batches made on the basis of disease symptoms may be inaccurate and are likely to vary depending on the conditions under which the seed is incubated. Also, symptomless seedling infection caused by *M. nivale* remains undetected using this method of estimating seed batch contamination by the pathogen. An alternative method is to plate surface sterilised seed onto agar (usually PDA) and record the number of seeds from which *M. nivale* colonies can be recovered after a period of incubation (Anon, 1985). This method is accepted by the International Seed Testing Association (ISTA) as a means of determining the incidence of *M. nivale* infection in seed. However, this method requires trained personnel who are able to identify accurately the fungal species present and it does not take into account inoculum which is on the seed surface. The plating of non-surface sterilised seed can lead to the underestimation of *M. nivale* contamination as the pathogen is readily out competed by other micro-organisms on the seed surface as well as *F. culmorum* (Pettitt *et al.*, 1993). These two methods depend on a period of incubation (usually 7 days) and accurate identification of disease symptoms or fungal cultures which can be time consuming particularly when several seed batches are to be tested. Estimations of the severity of *M. nivale* contamination of seed batches based on the percentage of infected

seeds consider only the incidence of infection and do not take into account the severity of contamination or infection on individual seeds. Malalasekera and Colhoun (1969) reported that the diameter of colonies of *F. culmorum* resulting from plating infected, surface sterilised seeds on a selective media at 25°C for five days was greater for seeds which were severely contaminated by the pathogen than for those which were less so. The authors also recorded agreement between the determination of the severity of seed naturally infected by *F. culmorum* and the incidence of seedlings which showed stem-base symptoms following growth in unsterilised soil for 27 days in a mean air temperature of 18.2°C. Hewett (1983) and later Humphreys *et al.* (1995) both reported a negative correlation between the percentage of seeds infected with *M. nivale* as determined by agar plate counts and the emergence of seedlings when the same seed was sown in the field. Low emergence scores were recorded for seed lots which had a high incidence of seeds infected with *M. nivale* however, both authors based their assertions on relatively few seed batches.

Correlations between the occurrence of pink grains and infection by *F. graminaerum* and *F. culmorum* were reported by Bechtel *et al.* (1985) and Perkowski and Chelkowski (1991) respectively. In contrast, Wakulinskiňski and Chelkowski (1993) and Hare (1997), were able to recover *Fusarium* spp. and *M. nivale*, respectively, from wheat grains which showed visual signs of contamination as well as those which did not. The visual appearance of seed, therefore, appears to be an unreliable method of estimating the severity of seed batch contamination by *Fusarium* spp. or *M. nivale.*

PCR allows all of the inoculum of a specific pathogen(s) present *in planta* to be detected and should not be influenced by the conditions, length of incubation or the presence of other micro-organisms. As with the agar plate method of detection, the technique requires trained staff. Quantification using competitive PCR assays allow comparisons on the degree of contamination between samples to be made. A rapid method of determining the severity of the infection of barley seed by *Pyrenophora* spp. was cited by Bates *et al.* (2001) as being a major advantage of replacing agar plate counts with

quantitative PCR assays providing the method delivered equivalent results to the agar plate count technique. Quantitative PCR also allows the determination of the degree of infection of fungal biomass between seed lots and individual seeds to be made whereas the agar plate count technique only determines the incidence of infection.

The objectives of the work presented in this chapter were (i) to determine the relationship between *M. nivale* infection of wheat seed determined by PDA plate counts and amount of *M. nivale* DNA determined using quantitative PCR assays from seed lots obtained from the 1997, 1998 and 1999 harvests. (ii) determine the relationship between estimates of infection using the two methods and seedling blight disease on seed from the 1999 harvest.

MATERIALS AND METHODS

RELATIONSHIP BETWEEN THE INCIDENCE OF *M. NIVALE* INFECTION AND ITS QUANTIFICATION USING PCR

Source of seed and determination of incidence of M. nivale

Wheat seed was obtained from the Official Seed Testing Station (OSTS), East Craigs, Edinburgh over three years; 1997 harvest (48 samples), 1998 harvest (67 samples) and 1999 harvest (90 samples). A further 40 samples were also obtained from OSTS, Cambridge from the 1999 harvest. The incidence of *M. nivale* infection in each seed batch was determined at the OSTS which provided each batch using the agar plate count technique according to ISTA guidelines (Anon, 1985). The origin, variety and percentage infection for each seed batch for each year is given in Appendices 7 – 10.

DNA extraction and quantification of M. nivale *var.* nivale *and* M. nivale *var.* majus

DNA was extracted from a 10 g sample of each seed batch and diluted to 40 ng μl^{-1} (Chapter 2, page 40). Diagnostic PCR reactions containing diluted sample DNA and using ITS4 and ITS5 primers described by White *et al.* (1990) with an anneal temperature of 50°C, were performed. Reaction conditions were as described by White *et al.* (1990) and

were performed in 25µl volumes. PCR products were visualised following gel electrophoresis as described in Chapter 2 (page 41). The amount of *M. nivale* var. *nivale* and var. *majus* was determined in seed batches from 1997, 1998 and 1999 harvests and the amount of *Fusarium* spp. was determined for seed batches from 1999 using the quantitative PCR assays described in Chapter 3. Quantitative PCR reactions were performed in 50 µl volumes containing 10 µl of the relevant internal standard and 10 µl of sample DNA. PCR products were visualized following gel electrophoresis using a Gel Doc 1000 flourescent gel documentation system (Bio-Rad Laboratories Ltd). The intensity of PCR products was determined by analysis of unsaturated images using Molecular Analyst 1.5 software (Bio-Rad). The ratio in intensity of fungal to internal standard PCR product was determined for each sample. Any sample with too much target DNA was diluted and quantification repeated. Total DNA in each sample was determined by spectrophotometry (Chapter 2, page 41). The amount of pathogen DNA was converted to pg of fungal DNA per ng of total DNA using the standard curve for either *Fusarium* spp., *M. nivale* var. *nivale* or var. *majus* derived in Chapter 3. Each quantitative PCR result was based on two replicates of each reaction.

RELATIONSHIP BETWEEN ESTIMATES OF *M. NIVALE* INFECTION AND SEEDLING BLIGHT SYMPTOMS

Sowing of seed and assessment of seedling blight symptoms

Seed from the 1999 harvest was sown in seed trays (21 cm x 15.5 cm); one hundred seeds from each seed batch were placed (cleft downwards) in compost (John Innes No. 2 sieved through a 5 mm sieve) in five rows of 20 seeds. Compost (roughly 2.5 cm depth) was used to cover the seed. Seed trays were placed on benches in a polythene tunnel according to a fully randomised design and watered over-head until compost was visibly moist. Temperature was measured using a temperature data logger (Tiny Talk, UK) set to take a reading every 30 min. The mean temperature over the course of the experiment was

7.49°C (max 19.5°C, min –0.9°C). Daily mean, maximum and minimum temperatures over the course of the experiment are given in Appendix 11.

At the third leaf stage (GS13), seedlings were removed from the trays, and roots, seed coat and compost were removed from the seedlings. The severity of stem-base disease symptoms for each seed batch was determined using a disease index system similar to that described in Chapter 2 (page 38) with the exception that a further category for severely stunted and diseased seedlings was used. Seedlings in this category were given a score of four. A combined disease index was also produced incorporating those seeds that did not emerge; seedlings in this category were given a score of five.

Determination of rate of emergence

The rate of emergence for each seed batch was determined by counting the number of emerged seedlings in each tray daily until no seedlings emerged on five consecutive days. A value for the rate of emergence for each seed batch was expressed as the reciprocal of time to mean emergence in days ($\check{D}$) calculated as :-

Equation 2. (From Khah *et al.*, 1986).

$$\check{D} = \Sigma (D.n) / \Sigma n$$

Where n is the number of seedlings which emerged on day D, counted as the number of days from sowing.

STATISTICAL ANALYSIS

Data for incidence of *M. nivale* seed infection, final emergence, and symptom incidence was Logit transformed in order to obtain normal distributions. Disease index data was Asin transformed and quantitative PCR data for *Fusarium* spp., *M. nivale* var. *nivale* and var. *majus* was Log transformed in order to obtain normal distributions. Genstat 5 Version 4.1 (Lawes Agricultural Trust, Rothamsted, UK) was used in order to perform linear regression analyses to determine the relationship between the quantification of *M.*

nivale DNA and PDA plate counts for seed samples from the three years. Linear regression analysis was also used to determine the relationship between the four estimates of seedling blight disease (rate of emergence, final emergence, severity of visible symptoms and incidence of visible symptoms). Multiple linear regression was used to determine the relationship between the four disease parameters and quantification of *M. nivale* and *Fusarium* spp. as well as that between the disease parameters and the individual *M. nivale* sub-species. An accumulated analysis of variance was used to determine significance taking into account each variate. Previous studies indicated that *M. nivale* var. *majus* was significantly more pathogenic as a seedling blight pathogen than *M. nivale* var. *nivale*. In order to take into account the effect of the differences in pathogenicity of the two sub-species when considering the disease symptom severity caused, a constant was applied to the values for *M. nivale* var. *majus* DNA in order to obtain an adjusted value for *M. nivale* (Equation 3). The constant (1.34) was derived from pathogenicity studies involving isolates of *M. nivale* var. *nivale* and var. *majus* (Chapter 6).

Equation 3.

Adjusted *M. nivale* = Log ((var. *majus* DNA x 1.34) + (var. *nivale* DNA))

An accumulated analysis of variance was again used to determine significance between the adjusted *M. nivale* DNA and disease parameters considered taking into account each variate. Comparisons between the sum of the *Fusarium* spp. DNA, *M. nivale* var. *nivale* DNA and var. *majus* DNA and disease and also the incidence of *M. nivale* and disease were made using linear regression analysis.

RESULTS

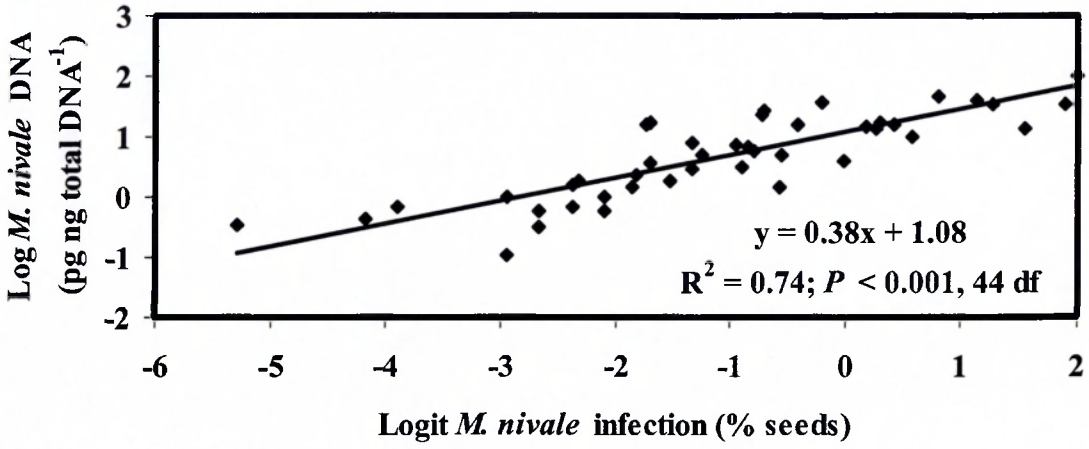
Incidence of seed infection and PCR quantification of M. nivale in seed

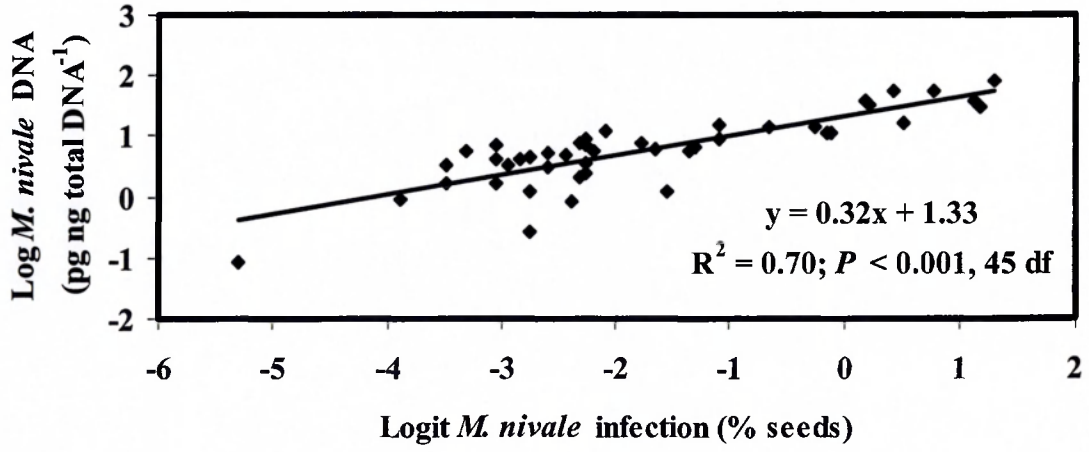
The incidence of *M. nivale* infection determined by the OSTS using agar plate counts ranged from 0.5% to 89.5% for samples from 1997, 0.5% to 78.5% for samples from 1998 and from 0.5% to 53.5% for samples from 1999 (Appendices 7 – 10).

PCR reactions using ITS4 and ITS5 primers resulted in a PCR product of expected size conforming to plant DNA for each sample. No evidence of PCR inhibition or excess genomic DNA was observed. The total amount of *M. nivale* DNA (*M. nivale* var. *nivale* DNA + *M. nivale* var. *majus* DNA) in seed samples where either one of the sub-species was detected ranged from 0.111 to 214.3 pg ng^{-1} total DNA for samples from the 1997 harvest. Total *M. nivale* DNA for samples from the 1998 harvest ranged from 0.258 to 81.668 pg ng^{-1}. Total DNA for samples from 1999 ranged from 0.089 to 113.9 pg ng^{-1} (Appendices 7 – 10).

Relationship between the incidence of M. nivale *seed infection and PCR quantification of* M. nivale *in seed*

Linear regression analysis between *M. nivale* incidence and PCR quantification showed that the relationship was positively correlated and highly significant ($P < 0.001$) for samples from each year (Figure 4.1). The variation accounted for by the regression was 74% in 1997 and 70% in 1998. The regression obtained for samples from 1999 was positively correlated for samples with percentage infections above 1.5%, with 70% of the variation accounted for by the regression. Samples with percentage infections below 1.5% were not positively correlated to quantification and when these were included in the regression analysis, the variation accounted for by the regression was 60%.





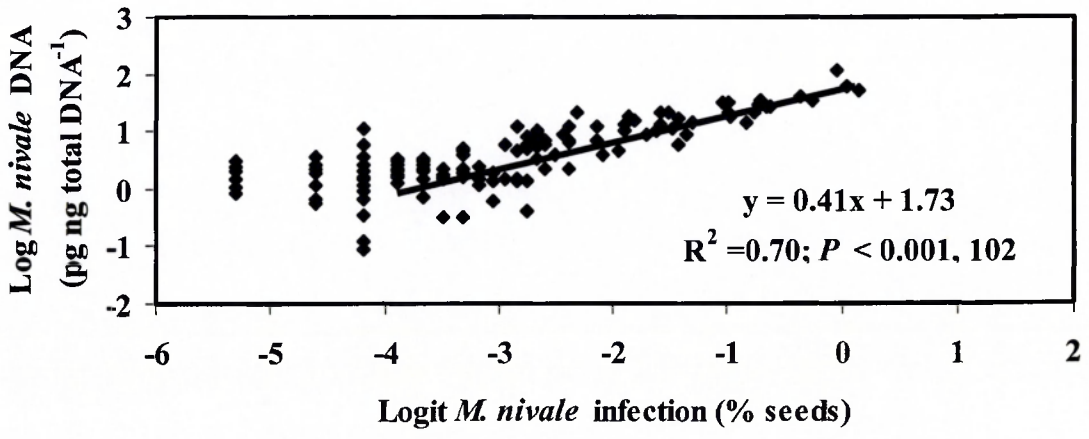


Figure 4.1. The relationship between *M. nivale* DNA and percentage infection for seed samples from (a) 1997, (b) 1998, (c) 1999 infected with *M. nivale*. Regression line for 1999 samples only incorporates samples with a percentage infection over 1.5%.

Quantification of* Fusarium *spp. in 1999 seed samples

Fusarium spp. were detected and quantified in all 130 seed samples from the 1999 harvest. The amount of *Fusarium* spp. DNA ranged from 8.00×10^{-3} to 11.19 pg ng^{-1} total DNA.

Relationship between estimates of* M. nivale *seed batch infection and seedling blight symptoms

Results for the accumulated analysis of variance comparisons between the estimates of pathogen infection: Fusarium DNA, *M. nivale* DNA, adjusted *M. nivale* DNA, var. *majus* DNA, var. *nivale* DNA and the incidence of *M. nivale* seed infection against the five disease parameters: stem-base disease severity, stem-base disease incidence, final emergence, combined disease severity (stem-base disease severity and final emergence) and rate of emergence are summarised in Table 4.1 – 4.4. With the exception of *M. nivale* var. *nivale*, estimates of pathogen contamination were significantly ($P = 0.001$) correlated to stem-base disease observed in the resulting seedlings (Table 4.1). The variation accounted for by the regression was greatest for disease severity when *Fusarium* spp., *M. nivale* var. *nivale* and var. *majus* DNA were considered separately (58.8%) and least using the sum of pathogen DNA (38.6%).

A highly significant ($P < 0.001$) correlation was observed between symptom incidence and all variates with the exception of *M. nivale* var. *nivale* DNA (Table 4.2). The variation accounted for was greatest for *M. nivale* incidence (50.7%) and least using the sum of pathogen DNA (36.8%). When *Fusarium* spp. DNA was compared to symptom incidence using multiple regression, a similar correlation was obtained ($R^2 = 47.4\%$) to that observed when total *M. nivale* and *Fusarium* spp. DNA was used ($R^2 = 46.9\%$). The sum of *Fusarium* spp. and total *M. nivale* DNA accounted for least variation in symptom severity ($R^2 = 36.8\%$).

Table 4.1. Summary of accumulated analysis of variance for regressions of visible seedling blight disease (disease index) observed on seedlings produced from 100 seeds from each of 130 seed batches of wheat from the 1999 harvest sown in compost, against amount of seed-borne pathogen DNA (determined using JBM, JBF, EFNiv or EFMaj quantitative PCR assays) or incidence of *M. nivale* infection in seed batches determined by PDA plate counts for seed from each of the seed batches.

Variate	V.R.	*P*	R^2	Total D of F
M. nivale DNA	95.6	<0.001	50.3	127
Fusarium spp. DNA	11.26	0.001	50.3	127
Adjusted *M. nivale* DNA	99.1	<0.001	51.0	127
M. nivale var. *majus* DNA	45.0	<0.001	58.8	98
M. nivale var. *nivale* DNA	3.6	0.060	58.8	98
Sum of pathogen DNA	82.0	<0.001	38.6	129
Incidence of *M. nivale* (% Infection)	166.9	<0.001	56.3	129

Table 4.2. Summary of accumulated analysis of variance for regressions of visible seedling blight disease incidence observed on seedlings produced from 100 seeds from each of 130 seed batches of wheat from the 1999 harvest sown in compost, against amount of seed-borne pathogen DNA (determined using JBM, JBF, EFNiv or EFMaj quantitative PCR assays) or incidence of *M. nivale* infection in seed batches determined by PDA plate counts for seed from each of the seed batches.

Variate	V.R.	*P*	R^2	Total D of F
M. nivale DNA	78.6	<0.001	46.9	126
Fusarium spp. DNA	16.5	<0.001	46.9	126
Adjusted *M. nivale* DNA	80.7	<0.001	47.4	126
M. nivale var. *majus* DNA	30.0	<0.001	50.5	97
M. nivale var. *nivale* DNA	1.6	0.215	50.5	97
Sum of pathogen DNA	75.6	<0.001	36.8	128
Incidence of *M. nivale* (% Infection)	132.4	<0.001	50.7	128

With the exception of *Fusarium* spp. DNA ($P = 0.637$), all variates were significantly ($P < 0.001$) correlated with final emergence (Table 4.3). The variation accounted for by the regression was greatest when *Fusarium* spp. DNA, *M. nivale* var. *nivale* DNA and var. *majus* DNA were considered separately to final emergence (45.0%) and least when compared to the sum of pathogen DNA (22.2%).

Combined disease severity was significantly ($P < 0.001$) correlated to all variates with the exception of *Fusarium* spp. DNA ($P = 0.067$) and *M. nivale* var. *nivale* DNA ($P = 0.015$) (Table 4.4). The variation accounted for was greatest when *Fusarium* spp. and *M. nivale* var. *nivale* and var. *majus* DNA were considered separately (59.3%) and least for the sum of pathogen DNA (34.0%). Rate of emergence was significantly correlated only to *Fusarium* spp. DNA ($P = 0.046$) and the sum of pathogen DNA ($P = 0.036$) though the variation accounted for was extremely low for both comparisons (3.0% and 2.6% respectively).

Table 4.3. Summary of accumulated analysis of variance for regressions of the number of emerged seedlings from 100 seeds from each of 130 seed batches of wheat from the 1999 harvest sown in compost, against amount of seed-borne pathogen DNA (determined using JBM, JBF, EFNiv or EFMaj quantitative PCR assays) or incidence of *M. nivale* infection in seed batches determined by PDA plate counts for seed from each of the seed batches.

Variate	V.R.	*P*	R^2	Total D of F
M. nivale DNA	45.8	<0.001	29.2	125
Fusarium spp. DNA	0.2	0.637	29.2	125
Adjusted *M. nivale* DNA	47.4	<0.001	29.8	125
M. nivale var. *majus* DNA	23.4	<0.001	45.0	97
M. nivale var. *nivale* DNA	6.1	0.015	45.0	97
Sum of pathogen DNA	37.3	<0.001	22.2	127
Incidence of *M. nivale* (% Infection)	62.0	<0.001	32.5	127

Table 4.4. Summary of accumulated analysis of variance for regressions of seedling blight disease severity (non-emerged and emerged seed combined) from 100 seeds from each of 130 seed batches of wheat from the 1999 harvest sown in compost, against amount of seed-borne pathogen DNA (determined using JBM, JBF, EFNiv or EFMaj quantitative PCR assays) or incidence of *M. nivale* infection in seed batches determined by PDA plate counts for seed from each of the seed batches.

Variate	V.R.	*P*	R^2	Total D of F
M. nivale DNA	77.4	<0.001	42.1	127
Fusarium spp. DNA	3.4	0.067	42.1	127
Adjusted *M. nivale* DNA	80.7	<0.001	43.0	127
M. nivale var. *majus* DNA	45.3	<0.001	59.3	98
M. nivale var. *nivale* DNA	6.0	0.016	59.3	98
Sum of pathogen DNA	67.4	<0.001	34.0	129
Incidence of *M. nivale* (% Infection)	122.9	<0.001	48.6	129

Relationships between disease parameters

Linear regressions between the seedling blight disease parameters are given in Figure 4.2). Disease severity was significantly ($P < 0.001$) correlated to both symptom incidence and final emergence with 82.7% and 52.4% respectively of the variation being accounted for by the regression lines obtained. Disease severity was also significantly ($P = 0.01$) correlated to rate of emergence though the variation accounted for by the regression line obtained was low (3.9%). Symptom incidence was significantly ($P < 0.001$) correlated with final emergence and combined disease severity, the variation accounted for by the regressions was 26.5% and 54.5% respectively. Symptom incidence was significantly ($P = 0.022$) correlated with rate of emergence though the variation accounted for was low (3.3%). Comparisons were not made between combined disease severity, disease severity or final emergence as these were used to derive the values for combined disease severity.

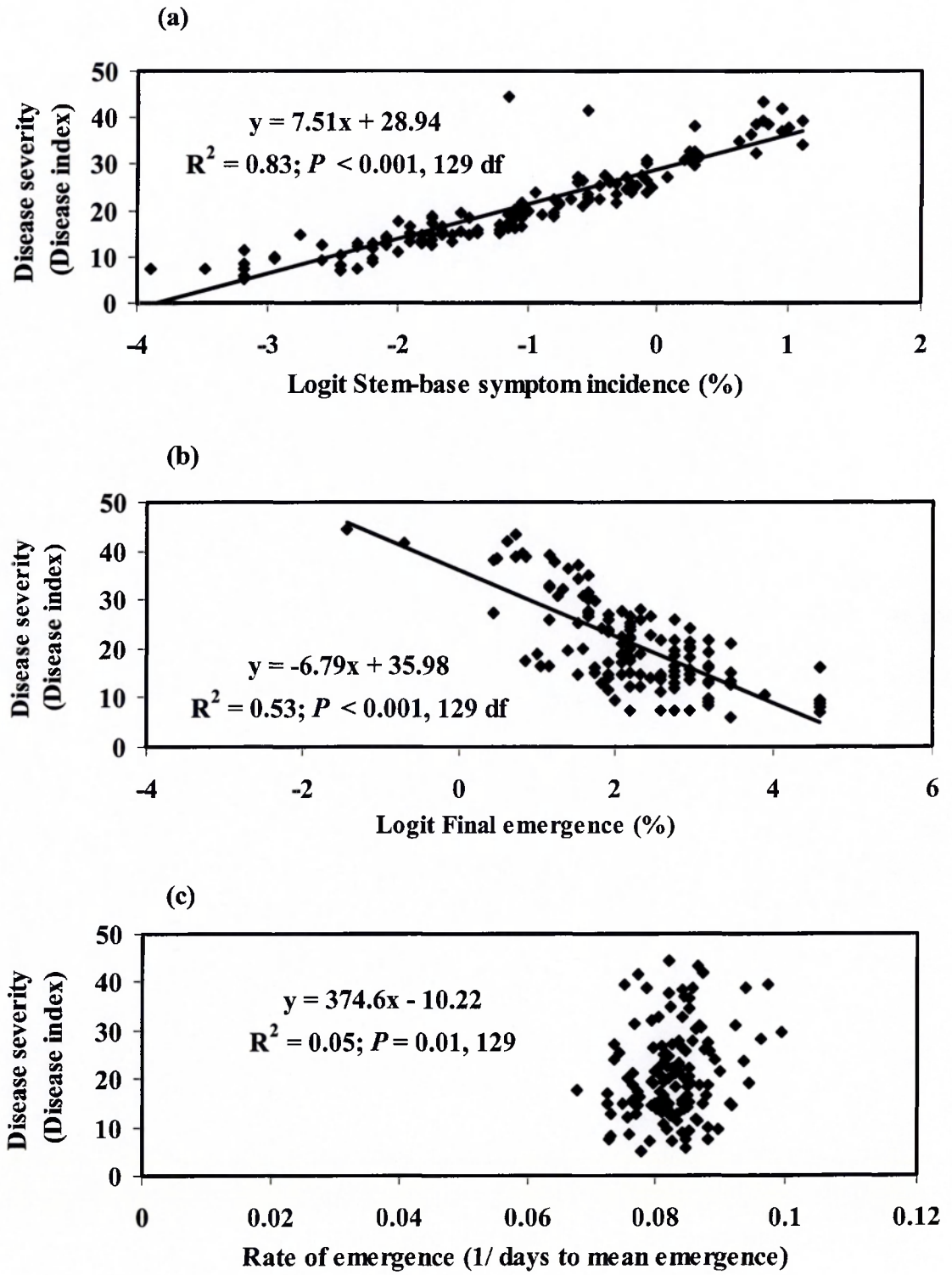


Figure 4.2. Relationship between seedling blight disease severity and (a) symptom incidence, (b) number of emerged seedlings and (c) rate of seedling emergence for seedlings produced from 100 wheat seeds from 130 seed batches from the 1999 harvest sown in compost.

DISCUSSION

The relationship between the incidence (plate counts) of *M. nivale* infection and quantitative PCR showed positive correlations which were highly significant. The regression, accounting for at least 70% of the variation within the data in each year. In a comparison of PCR and agar plate count techniques for the detection of *M. nivale* in infected seed lots Cockerell *et al.* (2001) reported that 95% of the variability in the regression was accounted for. The relationship they obtained was based on eight samples of which only two were below the 5% advisory threshold for drilling untreated seed. In contrast to the present study, the authors used the competitive PCR product ratio obtained for each sample and did not attempt to quantify pathogen DNA or take into account the variablility in the efficiency of DNA extraction in diluted sample DNA. In a comparison of methods for the detection of seed-borne *M. nivale*, Hare (1997) reported no significant difference between the recovery of *M. nivale* from seed on agar plates and the incidence of abnormal seedlings produced from seed incubated in paper towels. The results presented may indicate that in the majority of samples, *M. nivale* inoculum quantified using the PCR assays was situated within the seed coat and therefore not removed by surface sterilisation of the seed prior to plating onto PDA. A major factor affecting the variability which was not accounted for could be the range of *M. nivale* DNA on and within individual seeds. The relationship between *M. nivale* DNA and incidence altered for samples with percentage infections below 1.5% from 1999, no positive correlation was evident as was the case for samples with percentage infections above 1.5%. Samples received from 1999 had generally lower incidence of contamination by *M. nivale* probably due to Fusarium ear blight being less severe in 1999 (S.G. Edwards pers. comm.). A large proportion of samples had percentage infections of 1.5 % for which there was a large variation in inoculum detected by quantitative PCR assays. This effect is probably due to (i) variability in the amount of inoculum present on individual seeds, this cannot be measured by PDA

plate counts but is reflected in the variability of the PCR quantification (ii) variability in the PDA plate count results between seeds examined by PCR and those used for PDA plate count; the PDA plate count results were based on the examination of 200 seeds for each sample. For samples with low percentage infections the influence of each seed detected on the final result is great (50% for percentage infection of 1%). As the number of seeds infected rises the error caused by the mis-detection of a single seed falls as the final result is created by the mean of many infected seeds. Inaccuracies of PDA plate count results may also in part be due to competition from other seed-borne fungi during incubation thus leading to inaccurate estimations of percentage infections. Pettitt *et al.* (1993) reported that the recovery of *M. nivale* from the stem-bases of wheat plants could be inaccurate due to competition from *F. culmorum* and may in part account for the variability observed in this experiment. Bateman (1983) showed that the recovery of *M. nivale* from the outer epidermis, inner pericarp and testa of seed from one infected wheat seed batch but not another batch was significantly reduced when phenyl mercury acetate was applied to the seed surface. The recovery of *M. nivale* from the endosperm and embryo was unaffected by phenyl mercury acetate for either of the seed batches tested. Such variability may have been due to the inconsistency of surface-borne inoculum of *M. nivale* between seed lots. Further evidence for the inconsistency of superficial inoculum of *M. nivale* between seed batches was recorded by Hare (1997). He compared the incidence of *M. nivale* determined by agar plate counts from naturally infected seed which was largely free from fungal contaminants and had been surface sterilised, to seed from the same batch which had not been surface sterilised. Hare (1997) reported no obvious trend between seed samples; in at least one of the six seed lots tested, the incidence of *M. nivale* was greater, reduced or unchanged.

Bates *et al.* (2001) investigated the application of the PCR detection and quantification of *Pyrenophora* spp. in barley seed samples as a means of replacing the traditional agar plate method of determining the severity of seed batch contamination.

They produced seed batches with expected percentage infections ranging from 0 to 10 % by blending infected and healthy seed in different amounts. Although they achieved a correlation of r^2 = 0.931 between agar plate detection and PCR quantification of the pathogen, they also reported considerable variability between the expected and actual percentage infection values obtained. They concluded that this was a feature of the seed itself and not the technique used to measure infection. Bates *et al.* (2001) also compared percentage infection scores to PCR quantification for sixty samples received by OSTS for advisory testing and achieved a correlation of r^2 = 0.883 between the two techniques. They did however, report considerable variability in PCR quantification for samples with percentage infections above 40%. The authors attributed the variability in the amount of pathogen DNA in the samples to variability in the amount of inoculum per seed and not DNA extraction or PCR procedures. The results from this study suggest that variability in the amount of inoculum per seed exists which is most evident in seed batches with low percentage infections. The contradiction to the findings of Bates *et al.* (2001) may be due to differences in the epidemiology between *Pyrenophora* spp. infection of barley and *M. nivale* infections of wheat.

PCR identification of pathogens in plant material has the ability to detect inoculum which is both viable and non-viable whereas plate counts assess fungal inoculum which is able to grow and is therefore viable. A good correlation was obtained between PCR and plate count detection of *M. nivale* and PCR detection of *M. nivale* var. *nivale*, var. *majus*, *Fusarium* spp. and seedling blight disease. It would appear that the majority of Fusarium seedling blight inoculum present in seed samples and detected by PCR is indeed viable. The relationships between the estimates of *M. nivale* contamination and the parameters of disease showed several significant relationships indicating, in agreement with the findings of Hare (1997), that the severity of seed batch contamination by *M. nivale* influences the severity of seedling blight disease in the resulting seedlings. Although the variation

accounted between the estimate of *M. nivale* contamination and disease was variable depending on the method employed, all were useful augurs of seedling blight symptoms.

PCR quantification of *Fusarium* spp., *M. nivale* var. *nivale* and *M. nivale* var. *majus* showed better correlations to seedling disease than incidence of *M. nivale* though this was generally only evident when the amounts of the three pathogens were considered separately in the accumulated analysis of variance. The results support the conclusions of Colhoun (1970), who stated that seed-borne *F. avenaceum, F. culmorum* and *F. graminearum* is not a significant cause of pre-emergence seedling death at temperatures below 8°C. Colhoun and Park (1964) reported that pre-emergence death caused by *F. avenaceum*, *F. culmorum* and *F. graminearum* was more severe at 22.6°C than 17.6°C whereas the occurrence of stem-base lesions was greatest at the lower temperature. The results from the present work would appear to be in keeping with the findings of Colhoun and Park (1964). *Fusarium* spp. were less pathogenic than *M. nivale*; a poor correlation was obtained between *Fusarium* spp. fungal biomass and pre-emergence seedling death probably due to unfavourable temperatures, however plants that survived showed severe stem-base infection and this was significantly related to the amount of *Fusarium* spp. within the seed. Similarly, *M. nivale* var. *majus* appeared to be more pathogenic as a seed-borne pathogen of wheat than *M. nivale* var. *nivale*. When the difference in pathogenicity was incorporated in to the adjusted *M. nivale* DNA concentration values this resulted in an improvement of the variation accounted for, for disease severity and incidence, emergence and combined disease severity. This is in agreement with the findings of previous workers who inoculated leaves (Diamond and Cooke, 1999) and stem-bases (Simpson *et al.*, 2000), though contradicts the limited information given by Maurin *et al.* (1993) who showed that isolates of *M. nivale* var. *nivale* were more pathogenic than *M. nivale* var. *majus*. In contrast to *Fusarium* spp., *M. nivale* var. *nivale* caused reduced seedling emergence though was not significantly related to stem-base disease symptoms in this study. Although significant correlations between *Fusarium* spp. DNA and the rate of seedling emergence

described by Khah *et al.* (1986) were evident, the variation accounted for was low and none of the other estimates of the severity of infection resulted in significant correlations. Hare (1997) reported a significant association between the severity of *M. nivale* infection determined by agar plate counts and the thermal time to mean emergence. However, Hare (1997) used only six seed lots. It would appear, therefore, that the rate of seedling emergence is influenced more by temperature than the severity of seed batch contamination.

The incidence and severity of seedling blight disease symptoms were highly correlated as were final emergence and stem-base disease severity under the conditions employed. Humphreys *et al.* (1995) reported that in field studies and in trays of compost, seedlings that survived pre-emergence mortality caused by *M. nivale* or *Fusarium* spp. were largely free from infection following establishment. Hare (1997) reported reductions in emergence of 70% due to seed-borne *M. nivale* in experiments performed at 6°C; all surviving seedlings showed stem-base symptoms. When the same seed was sown at 12°C, emergence was reduced by only 20% and only 40% of surviving plants showed visual symptoms which were less severe than those on seedlings grown at 6°C. Severe symptoms were also reported on seedlings which survived pre- and post-emergence death caused by *F. graminearum* (Kane and Smiley 1987). The results from this study showed that seed lots with the lowest emergence scores had more severe stem-base symptoms and are in keeping with the findings of both Hare (1997) and Kane and Smiley (1987). The contradiction to the findings of Humphreys *et al.* (1995) may be a result of the detection of both symptom-causing and symptom-less pathogen infections in this study.

The determination of seed batch contamination by agar plate counts and PCR quantification were in general agreement for samples from the three years tested. PCR quantification of pathogen inoculum was as good and often improved predictor of seedling disease compared to the agar plate count technique. Analysis of infected seed samples does however require more expensive equipment and consumables compared to the plate

count method. Skilled staff are required for both PCR analysis and plate count analysis however staff trained in PCR analysis could also be used for other molecular work. The quantitative PCR assays described here fulfills the requirements of a seed health test described by Bates *et al.* (2001) that it should be a rapid method and deliver equivalent results to the agar plate technique.

This study has demonstrated that the quantification of seedling blight pathogens using competitive PCR assays could provide a useful alternative to the current plate count method for determining the severity of seed lot contamination. Worthy of note is that from receipt of a grain sample to a final quantitative PCR result can be achieved within three days compared to a minimum of seven days using plate counts. Though correlations between seed batch contamination by seedling blight pathogens and seedling blight disease were observed, had the experiment been performed under different conditions, disease severity may have altered. Further work could focus on the relationship between contamination by *M. nivale* var. *nivale*, var. *majus*, *Fusarium* spp. and seedling blight symptoms under different environmental conditions and for example in the field, with varied drilling times. This may facilitate the production of a comprehensive predictive model as to the severity of Fusarium seedling blight under field conditions. Other further work could involve the quantification of pathogen inoculum on the surface of individual seeds and its relevance to seedling blight disease particularly those from seed batches with low percentage infections in order to determine the amount of inoculum which remains undetected by plate counts.

CHAPTER 5

ANNUAL AND REGIONAL DISTRIBUTIONS OF *M. NIVALE* VAR. *NIVALE* AND VAR. *MAJUS* WHEAT IN UK SEED BATCHES

INTRODUCTION

Studies such as those described by Wollenweber and Reinking (1935) and Lees *et al.* (1995), have resulted in a greater understanding of the taxonomy within *M. nivale*. Few epidemiological studies have been performed with respect to annual variations and regional distributions of the two sub-species of *M. nivale* (var. *majus* and var. *nivale*) within seed samples. Studies have been reported that highlight regional and annual variations in the incidence of *M. nivale* and species of *Fusarium* on wheat crops in the UK. Polley and Turner (1995) reported both annual and regional variations in pathogens associated with stem-base and ear blight diseases of wheat in England, Wales and Scotland between 1989 and 1990. *Microdochium nivale* was more common than any species causing Fusarium stem-base disease in the UK in both 1989 and 1990, however, the pathogen accounted for 73% of the isolates identified from Scottish samples in 1989 and only 41% in 1990. In the South East, Midlands and North East of England, *M. nivale* was most common in 1989, although *F. culmorum* was the most common pathogen isolated in 1990. The incidence of Fusarium ear blight was greatest in Wales and the West in 1989, however, in 1990, the disease was most common in East Anglia.

Reeves and Wray (1994) summarised results from the National Institute of Agricultural Botany (NIAB) seed certification and advisory seed testing programme. They reported a close correlation between the increase in *M. nivale* infection in seed batches and the number of seed batches with poor germination. No regional data was available for *M. nivale* infection. In 1991 however, when the South West of England experienced wet, humid weather up to harvest, compared to the rest of England and Wales, germination was much reduced in seed batches received from this region. In 1992, when wet weather was general throughout the country, lower germinations were recorded from seed batches received from throughout England and Wales. This result corroborated the findings of Hyde (1950) who reported that the amount of sub-epidermal fungi present in wheat grains from 27 world sites was greatest in regions where humidity was high in the weeks

preceding harvest. Cockerell and Rennie (1996) recorded annual and regional variations in the severity of *M. nivale* infection in winter wheat seed from England and Scotland between 1992 and 1994. In 1992 and 1993, 99% of all seed batches tested were infected with *M. nivale*, of which 41% in 1992 and 59% in 1993, of English samples had percentage infections of greater than 20%. Approximately 20% of Scottish samples however, had percentage infections greater than 20% in both years. In 1994, only 68% of samples received were infected by *M. nivale* of which 1% of English samples and 7% of Scottish samples were above 20% infection.

The studies reported so far have highlighted the annual and regional variations in Fusarium disease or the contamination of seed samples by *M. nivale*, however, none of these studies reported on the identification of sub-species within *M. nivale*. Both Parry *et al.* (1995b) in the UK and Nirenberg *et al.* (1994) in Germany reported that *M. nivale* var. *majus* was more common on wheat seed samples than var. *nivale*. Parry *et al.* (1995b) used the presence of the *Rsa* I restrictions site within rDNA first described by Maurin *et al.* (1995) to distinguish isolates of variety *majus* from those of variety *nivale* for strains isolated from the stem-bases and seed of UK wheat samples. Of 144 stem-base isolates from around the UK, 70% were var. *majus* and 30% var. *nivale*, whereas 93% of 91 grain isolates from around the UK were var. *majus* and 7% var. *nivale*. Mahuku *et al.* (1998) used the same method as Parry *et al.* (1995b) to sub-speciate 100 isolates of *M. nivale* from turf grass taken from four sites in Ontario, Canada. All isolates lacked the *Rsa* I restriction site and were termed var. *nivale*. Although the studies by Parry *et al.* (1995b) and Mahuku *et al.* (1998) were the first which examined the occurrence of the two *M. nivale* sub-species in the field, neither study took into account the amount of inoculum present within individual samples.

The objectives of the work presented in this chapter are to (i) use the quantitative PCR assay described in Chapter 3 to quantify *Fusarium* spp. in seed samples from 1997 and 1998 and use these data together with the quantitative PCR data generated in Chapter 4

to (a) determine any association in the occurrence of *M. nivale* var. *nivale*, var. *majus* and *Fusarium* spp. in English and Scottish samples from 1997, 1998 and 1999 harvests (b) determine the relationship between *M. nivale* var. *nivale*, var. *majus* and *Fusarium* spp. inoculum in seed batches from England and Scotland between 1997 and 1999 (ii) determine the predominant *M. nivale* sub-species in England and Scotland in the same samples.

MATERIALS AND METHODS

QUANTITATIVE PCR DATA AND THE ASSOCIATION BETWEEN *M. NIVALE* VAR. *NIVALE*, VAR. *MAJUS* AND *FUSARIUM* SPP.

The quantitative PCR assay for *Fusarium* spp. described in Chapter 3 (page 41) was used to quantify *Fusarium* spp. in seed samples from 1997 and 1998. DNA previously extracted from each 1997 and 1998 seed batch as described in Chapter 4 was used, the methodology for quantification of *Fusarium* spp. using competitive PCR followed that described in Chapter 3 (page 41). The incidence of detection of *M. nivale* var. *nivale*, var. *majus* and *Fusarium* spp. using the PCR assays with seed samples from England and Scotland from the three years was recorded in order to determine any associations between the occurrence of the pathogens within the seed samples. Positive and negative PCR signals obtained using each of the three primer sets for each seed sample were recorded. Results were split into two groups based on the country of origin of the seed sample (i.e. Scotland or England) in each year (Appendices 7-10) for analysis.

STATISTICAL ANALYSIS

Relationships between pathogens

Quantitative PCR data were Log transformed to obtain normal distributions, Genstat 5 release 4.1 (Lawes Agricultural Trust, Rothamsted, UK) was used for regression analysis between the amount of *M. nivale* var. *nivale* and var. *majus* in seed batches in each year. Regression analysis was also used for comparisons between *Fusarium* spp. and *M. nivale* var. *nivale* and var. *majus* DNA for each year's samples.

Determination of pathogen predominance

To determine the predominant *M. nivale* sub-species in each seed batch, the ratio between the amounts of the two pathogens was used, (Amount of *M. nivale* var. *nivale*/ Amount of *M. nivale* var. *majus*). Samples in which either one or neither of the sub-species could be detected were removed from the data set. The pathogen ratio data were Log transformed in order to obtain normal distributions. Genstat 5 release 4.1 was used in order to produce a general linear model with year (1997, 1998 or 1999) and country (Scotland or England) as factors, which were then analysed using an accumulated analysis of variance. Fitted values were used in order to generate a predicted mean and standard error value for the pathogen ratio in each country for each year.

Effect of cultivar Riband on Scottish pathogen ratios

The cultivar Riband was the most common variety of winter wheat grown in Scotland and was the most common variety within the seed lots received from Scotland in each of the three years. A generalised linear model and accumulated analysis of variance was used to test the influence of this variety within the pathogen ratio data set from the Scottish samples over the three years. Factors for the model produced were year (1997, 1998 or 1999) and variety (Riband or all other varieties).

RESULTS

QUANTITATIVE PCR DATA AND THE ASSOCIATION BETWEEN *M. NIVALE* VAR. *NIVALE*, VAR. *MAJUS* AND *FUSARIUM* SPP.

Fusarium spp. were detected in all Scottish and English seed samples from 1997 and 1998. The amount of *Fusarium* spp. detected in Scottish samples from 1997 ranged from 1.99×10^{-5} to 0.41 ng pg^{-1} and for English samples from 2.35×10^{-4} to 0.24 ng pg^{-1}. For 1998 samples, the amount of *Fusarium* spp. detected in Scottish samples ranged from 0.15 to 105.6 ng pg^{-1} and for English samples from 0.19 to 67.44 pg ng^{-1}. *Fusarium* spp. were also detected in all samples from 1999 (Chapter 4).

Microdochium nivale var. *nivale* and var. *majus* were both detected in the majority of samples from each country in each year (Table 5.1). The frequency of detection of only one sub-species and the detection of neither sub-species was low for samples from both countries in 1997 and 1998. In 1999, *M. nivale* var. *majus* was detected independently of var. *nivale* in 11 English and 17 Scottish samples.

Table 5.1. Number of wheat seed samples where *M. nivale* var. *nivale* and var. *majus* was detected in English and Scottish seed samples in 1997, 1998 and 1999 harvests using diagnostic PCR.

Year	England				Scotland			
	Sub-species identified/ frequency				Sub-species identified/ frequency			
	n & m	n only	m only	Neither	n & m	n only	m only	Neither
1997	16	4	2	2	21	0	1	1
1998	31	1	0	0	14	0	0	1
1999	49	1	11	2	50	0	17	0

n = *M. nivale* var. *nivale*
m = *M. nivale* var. *majus*

RELATIONSHIP BETWEEN *M. NIVALE* VAR. *NIVALE* AND VAR. MAJUS

Regression analysis between the amount of *M. nivale* var. *nivale* and var. *majus* from the English and Scottish seed batches over the three years showed a positive relationship for each country in each year (Figures 5.1a – c). The relationship between the two sub-species was significant ($P < 0.05$) in all cases (Table 5.2). The level of significance ranged from $P = 0.002$ to $P < 0.001$ and the variation accounted for by the regressions ranged from 28.5% for English samples from 1999 to 71.7% for Scottish samples from 1997.

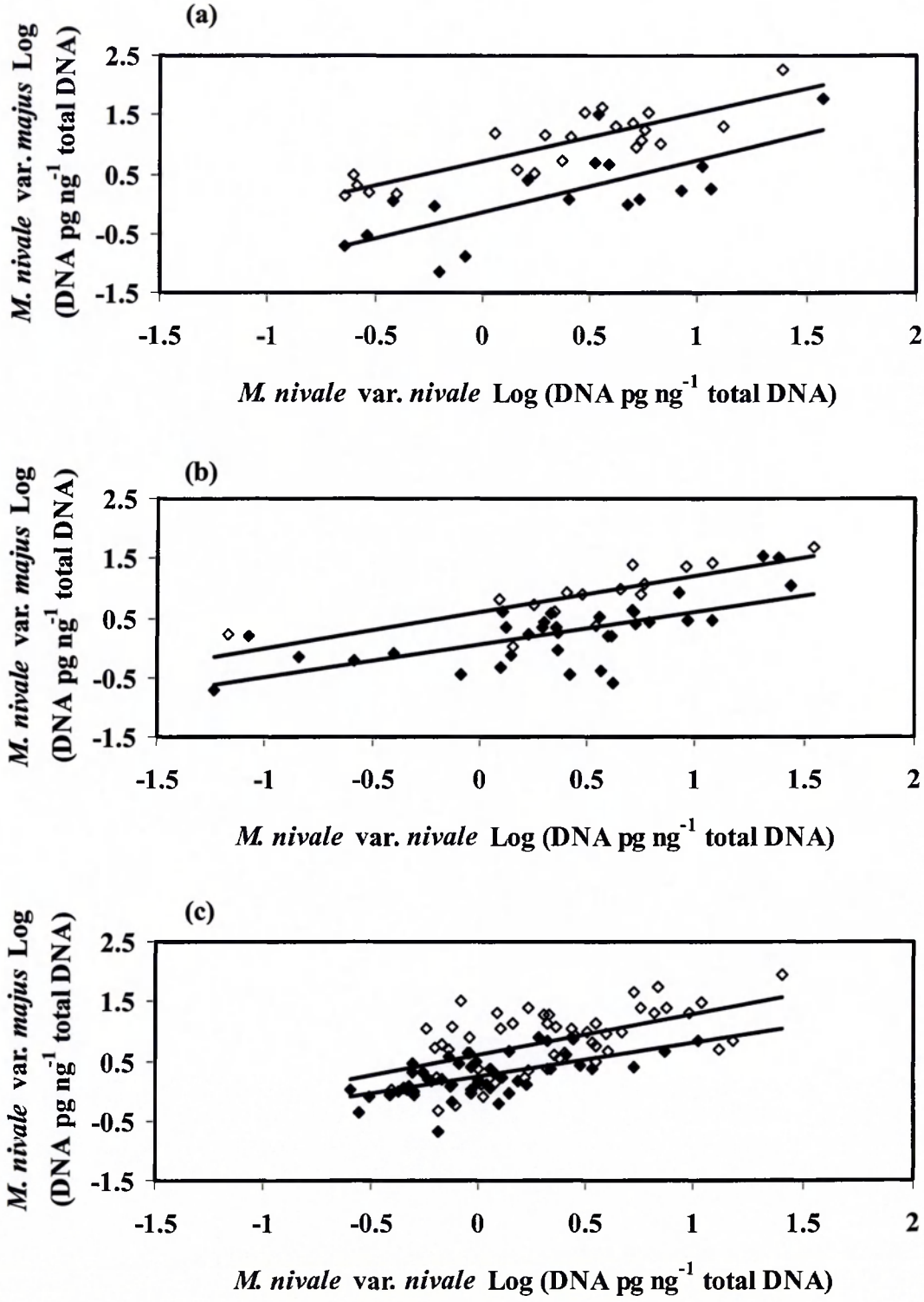


Figure 5.1. The relationship between quantity of *M. nivale* var. *nivale* and var. *majus* DNA in (◆) English and (◇) Scottish seed samples from (a) 1997, (b) 1998 and (c) 1999 harvests. Statistics for each regression line are given in Table 5.3.

Table 5.2. Summary of regression analyses for *M. nivale* var. *nivale* and var. *majus* for seed samples from Scotland and England harvested in 1997, 1998 and 1999.

Year	Country	F. Probability	Slope of regression	Standard error	R^2	D of F
1997	Scotland	<0.001	0.81	0.30	71.7	20
	England	0.002	0.87	0.56	48.5	15
1998	Scotland	<0.001	0.61	0.31	58.1	13
	England	<0.001	0.55	0.41	41.1	30
1999	Scotland	<0.001	0.57	0.27	35.3	48
	England	<0.001	0.68	0.44	28.5	49

Relationship between* M. nivale *var.* nivale, *var.* majus *and* Fusarium *spp.

The ANOVA Table for regression analysis between the amount of *Fusarium* spp., *M. nivale* var. *nivale* and var. *majus* is given in Table 5.3. The amount of *Fusarium* spp. DNA was significantly ($P = 0.012$) related to the amount of *M. nivale* var. *majus* DNA for Scottish samples in 1999 though the variation accounted for was low (8.0%) (Table 5.3). No significant ($P < 0.05$) relationship was observed between the amount of *Fusarium* spp. DNA and the amount of *M. nivale* var. *nivale* DNA for samples from any of the three years or between *Fusarium* spp. and *M. nivale* var. *majus* for samples from 1997 or 1998.

Table 5.3. Summary of regression analyses between quantity of *Fusarium* spp. DNA and *M. nivale* var. *nivale* and var. *majus* DNA for English and Scottish seed samples from 1997, 1998 and 1999 harvests.

Year	Country	Sub-species	F. Prob.	Slope	Standard Error	D of F	R^2 (%)
1997	Scotland	*nivale*	0.33	-0.15	0.59	20	0.1
		majus	0.84	-0.03	0.61	21	0.0
	England	*nivale*	0.13	-0.34	0.61	19	7.6
		majus	0.34	-0.28	0.78	17	0.0
1998	Scotland	*nivale*	0.34	0.23	0.62	13	0.0
		majus	0.53	0.12	0.49	13	0.0
	England	*nivale*	0.14	-0.25	0.63	31	4.0
		majus	0.16	-0.21	0.54	30	3.5
1999	Scotland	*nivale*	0.82	-0.024	0.42	49	0.0
		majus	0.01	-0.28	0.56	66	8.0
	England	*nivale*	0.41	0.06	0.35	49	0.0
		majus	0.16	0.11	0.40	59	1.7

Determination of pathogen predominance

The ratio between *M. nivale* var. *nivale* and *M. nivale* var. *majus* DNA obtained for seed samples from 1997 ranged from 0.08 to 0.67 for Scottish samples and 0.11 to 10.7 for English samples. For 1998 seed samples, pathogen ratios ranged from 0.04 to 1.43 for Scottish samples and 0.21 to 9.27 for English samples, for 1999 seed samples, pathogen ratios ranged from 0.03 to 2.66 for Scottish samples and from 0.18 to 3.24 for English samples.

The generalised linear model analysis and accumulated analysis of variance showed that the pathogen ratios were highly significant ($P < 0.001$) between years and also between countries of origin (Table 5.4). The significant difference was greatest between country than between year as indicated by the greater variance ratio for country (53.61) than year (8.53). A significant ($P < 0.001$) interaction was observed between country and year factors indicating that the trend differed between both year and country. The back transformed predicted means from the fitted values obtained from Scottish samples were 0.23 (1997), 0.40 (1998) and 0.32 (1999) and for English samples; 1.59 (1997), 1.46 (1998) and 0.58 (1999). Predicated means of greater than 1.00 indicate that *M. nivale* var. *nivale* was the predominant sub-species (Figure 5.2).

Table 5.4. Accumulated analysis of variance table for generalised linear model analysis of *M. nivale* var. *nivale/* var. *majus* DNA ratio for English and Scottish seed samples from 1997, 1998 and 1999 harvests.

Factor	D of F	V. R.	*P*
Year	2	8.53	<0.001
Country	1	53.61	<0.001
Interaction	2	7.58	<0.001
Residual	167		
Total	172		

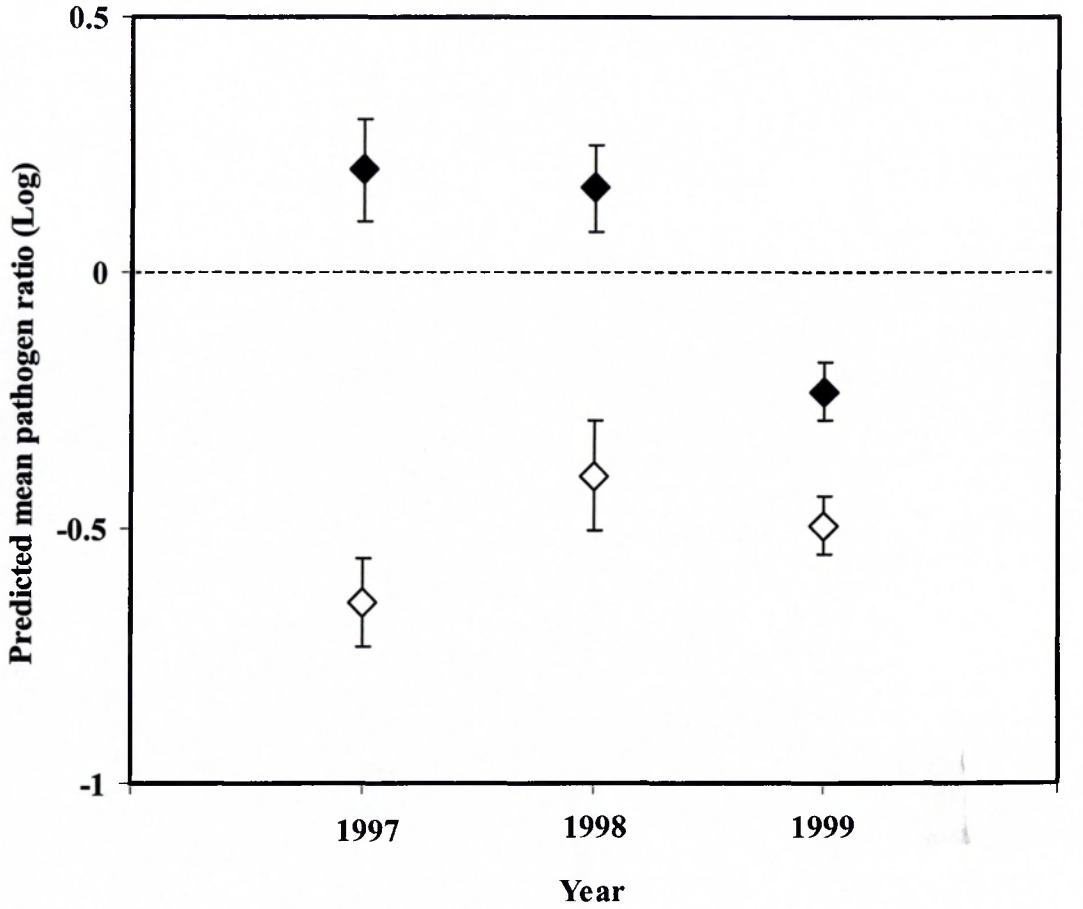


Figure 5.2. Predicted mean values obtained from generalised linear model analysis of *M. nivale* var. *nivale*/ var. *majus* DNA ratio for English and Scottish seed samples from 1997, 1998 and 1999 harvests (◆) England (◇) Scotland. Bars indicate standard errors. Logarithmic predicted mean pathogen ratio of greater than zero indicates *M. nivale* var. *nivale* dominant.

Influence of variety Riband on Scottish pathogen ratio data

The generalised linear model produced showed no significant ($P = 0.82$) difference between varieties (Riband or all other varieties) or years ($P = 0.20$). No significant ($P = 0.47$) interaction between year and variety was observed (Table 5.5). The back transformed predicted means from the fitted values obtained for Riband were 0.20 (1997), 0.53 (1998) and 0.32 (1999) and for all other varieties; 0.31 (1997), 0.35 (1998) and 0.33 (1999) (Figure 5.3).

Table 5.5. Accumulated analysis of variance table for generalised linear model analysis of *M. nivale* var. *nivale*/ var. *majus* DNA ratio for Scottish seed samples (variety Riband against all other varieties) from 1997, 1998 and 1999 harvests.

Factor	D of F	Variance ratio	F. Probability
Year	2	1.65	0.20
Variety	1	0.06	0.82
Interaction	2	0.76	0.47
Residual	76		
Total	81		

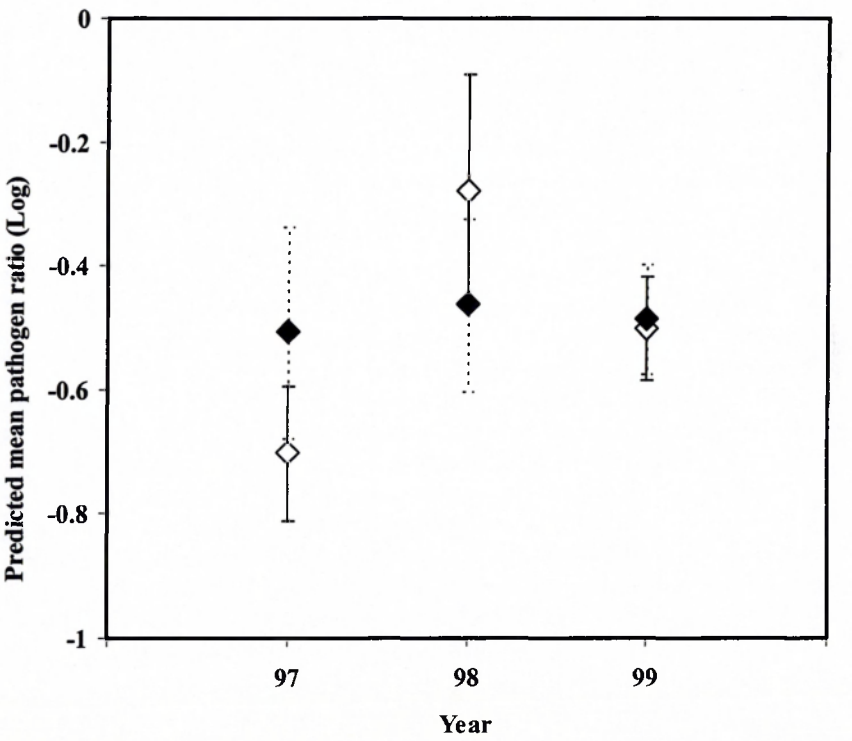


Figure 5.3. Predicted mean values obtained from generalised linear model analysis of *M. nivale* var. *nivale*/ var. *majus* DNA ratio for Scottish seed samples from 1997, 1998 and 1999 (◆) Riband (◇) all other varieties. Bars indicate standard errors. Logarithmic predicted mean pathogen ratio of greater than zero indicates *M. nivale* var. *nivale* dominant.

DISCUSSION

The majority of seed samples from each country in each year contained inoculum of both *M. nivale* var. *nivale* and *M. nivale* var. *majus*. Chi^2 analysis can be used to test for associations between the occurrences of entities within biological systems. A key parameter of the test is that there are at least five observations in each category. In the present study, fewer than five observations were present in at least one category for each year and from each country. Therefore, Chi square analysis could not be performed. The low number of observations of each sub-species occurring independently suggests that no exclusive competition between the two sub-species exists. Regression analysis indicated a positive relationship exists between the amount of each sub-species for samples from both England and Scotland. *Fusarium* spp. were detected in all samples tested and, the amount of *Fusarium* spp. was not correlated to the amount of *M. nivale* var. *nivale* or var. *majus* with the exception of *M. nivale* var. *majus* in Scotland in 1999. Comparison of pathogen ratios for English and Scottish samples between the three years showed that *M. nivale* var. *nivale* inoculum predominated in English samples in 1997 and 1998 and *M. nivale* var. *majus* inoculum was predominant in English samples from 1999. In Scotland, *M. nivale* var. *majus* was the predominant sub-species in all three years and accounted for a greater proportion of the *M. nivale* inoculum in Scottish samples from 1999 than it did in English samples obtained from the same year. Parry *et al.* (1995b) were able to isolate *M. nivale* var. *majus* from each of seven grain samples from around the UK, however, isolates identified as *M. nivale* var. *nivale* were distinguished from only three of the seed lots. In this study, the two *M. nivale* sub-species could be detected in the majority of grain samples in each year. No regional differences in the occurrence of the two sub-species were evident in the data presented by Parry *et al.* (1995b) as they used only seven seed batches only one of which was from Scotland. Using wheat stem-base samples from 30 English sites, the authors detected both *M. nivale* sub-species from 18 sites whereas variety *majus* only could be isolated from 8 sample sites and variety *nivale* only from 4 sample sites.

Results from the present study showed that both *M. nivale* sub-species were detected in the majority of samples from each year indicating that the two sub-species were commonly found together in grain samples. This distribution of sub-species on grain is similar to that found by Parry *et al.* (1995b) using stem-base samples. Further analysis of the results presented by Parry *et al.* (1995b) showed that more isolates of *M. nivale* var. *majus* than var. *nivale* were identified in stem-base samples from 21 sites. Variety *nivale* was more common than var. *majus* at 7 sites and the same number isolates of each sub-species identified in samples from 2 sites. In the present study, *M. nivale* var. *majus* generally predominated over var. *nivale* although regional differences were evident. Parry *et al.* (1995b) did not focus on regional differences between the occurrence of the two sub-species as they concluded that too many extraneous influences existed. However, of the 69 isolates of *M. nivale* which they identified from stem-base samples from sites in the North of England, 55 were *M. nivale* var. *majus* and 14 were var. *nivale,* whereas, of 75 isolates from sites in the South of England, 46 were var. *majus* and 29 var. *nivale.* This suggests that, in keeping with the findings of this study from 1999 samples of grain, var. *majus* appeared to predominate though was more dominant in samples from a more Northerly location than those from the South. Bardsley *et al.* (1998) reported a survey of stem-base pathogens present in 667 wheat samples from England and Scotland between 1997 and 1998. *Microdochium nivale* var. *nivale* was the dominant *M. nivale* variety present in samples from East Anglia, the Midlands, North East and South. The two sub-species were present in equal proportions in samples from the North West and that *M. nivale* var. *majus* was the dominant sub-species present in samples from Scotland and the Borders. The results from the present investigation using grain samples from the same period (1997 and 1998) from the same regions appear to corroborate the findings of Bardsley *et al.* (1998) with var. *nivale* predominating in samples from England whereas var. *majus* predominates in Scotland. It would, therefore, seem possible that the occurrence of *M. nivale* sub-species on the stem-base is a determinant factor in the occurrence of *M. nivale* sub-species

in the resultant grain. Parry *et al.* (1995b) suggested that the predominance of var. *majus* on wheat stem-bases, combined with its capacity for homothallic sexual reproduction may lead to a greater production of spores than for var. *nivale* resulting in a greater incidence of ear blight and seed infection caused by var. *majus*. Parry *et al.* (1995b) cited several extraneous influences that could affect conclusions made as to the regional differences between the occurrence of *M. nivale* sub-species in wheat samples. These factors which include climate, soil type, cropping history, source of seed, fungicide programme, cultivar and competition with other pathogens, are likely to influence the results of the present study to varying degrees. No evidence is available in the literature that soil type or fungicide programme influence the occurrence and severity of ear infection by the two *M. nivale* sub-species differently, though this does not serve to exclude these as influential factors. Bardsley *et al.* (1998) did show that *M. nivale* var. *nivale* was slightly more common in stem-base samples of wheat grown following cereals or broad leaved crops than *M. nivale* var. *majus*. The two sub-species were detected in the same proportions in wheat crops grown following grass, maize or onions and *M. nivale* var. *majus* was slightly more prevalent in wheat grown following set-aside. Bateman (1993) reported that *M. nivale* and both *T. acuformis* and *T. yallundae* co-occurred more frequently on the stem-bases of winter wheat crops sampled in the middle of July between 1989 and 1991 than was expected. Turner *et al.* (1999) reported that *M. nivale* var. *majus* was more commonly associated with both *T. acuformis* and *R. cerealis* on stem-base samples of wheat in 1993 than was anticipated by chance. West *et al.* (1998) reported that Scotland had a higher incidence and severity of stem-base infection of wheat caused by *T. acuformis* in 1998 and also a lower incidence and severity of infection caused by *T. yallundae* than in England. In England, though *T. acuformis* still predominated, the differences in the incidence and severity of infection was more similar and was lowest in samples from the South. Thus the predominance of *M. nivale* var. *majus* in grain samples from Scotland in 1997 and 1998 may be a result of the predominance of var. *majus* on the stem-base of wheat crops. This

in turn may be a result of the higher occurrence and severity of stem-base infection by *T. acuformis* which predominates in Scotland.

Riband was the most common variety of the grain samples received from Scotland, though pathogen ratios obtained for this variety did not differ significantly from that obtained for the other varieties. Kenyon and Thomas (2001) reported that *M. nivale* percentage infection declined with an increase in varietal Fusarium ear blight resistance rating. However, they failed to state whether the relationship was significant and the resistance rating accounted for only 5.8% of the variation between samples. The source of seed initially used, competition with other pathogens and climate would, therefore, appear to be the most influential factors accounting for the differences in the amount of *M. nivale* var. *nivale* and var. *majus* detected. No significant relationship was observed between the amount of either *M. nivale* sub-species quantified and the amount of *Fusarium* spp. quantified, with the exception of *M. nivale* var. *majus* in Scotland in 1999, when the variation accounted for was low. This suggests that no obvious direct competition existed between either *M. nivale* sub-species and the other major group of pathogens which are responsible for Fusarium ear blight and the contamination of UK grain. Ear infections of wheat caused by *F. graminearum* and *F. culmorum* are favoured by temperatures in the range 20 to 30°C (Sutton, 1982) and are optimal around 25°C (Andersen, 1948, Parry *et al.*, 1994), whereas, ear infections caused by *M. nivale* are favoured by lower temperatures (Parry *et al.*, 1994). *Microdochium nivale* and *Fusarium* spp. have also been shown to have different sensitivities to fungicides used to control ear blight pathogens (Jennings *et al.*, 2000). Thus the lack of a significant relationship between *M. nivale* and *Fusarium* spp. in the grain samples tested may also be a result of interactions between climatic conditions and fungicides used during grain production.

Kenyon and Thomas (2001) reported a correlation between the amount of *M. nivale* infection in seed samples received by the OSTS, Cambridge from separate English counties and rainfall between 9^{th} – 14^{th} of June between 1994 and 1999. The variation

accounted for was 53.4%. In 1997, counties which experienced high rainfall during the same period produced seed batches with high incidences of *M. nivale*, whereas in 1998 when a similar amount of rainfall was experienced, the incidence of *M. nivale* contamination was lower. They concluded that factors such as spring rainfall or temperature may also account or the seasonal differences in *M. nivale* infection levels. In 1999, both rainfall and the level of *M. nivale* infection were much reduced compared to the previous two years. Over the same period as that investigated by Kenyon and Thomas (2001), this study has shown that *M. nivale* var. *nivale* was the predominant sub-species present in English samples in 1997 and 1998, a period when rainfall was high between the 9th and 14th of June. In 1999, when rainfall was lower over or the same period, *M. nivale* var. *majus* was the predominant *M. nivale* sub-species in English samples. Daamen *et al.* (1991) reported that the contamination of Netherlands grain by *M. nivale* and *Fusarium* spp. was positively correlated with cumulative precipitation in June, July and August and negatively correlated with the average temperature in July and August. *Microdochium nivale* accounted for the greatest proportion of infected seeds at average temperatures of 15°C and the least proportion at temperatures greater than 17°C.

The application of diagnostic PCR for the detection of *M. nivale* var. *nivale* and var. *majus* in this study has shown that the two sub-species of *M. nivale* are usually detected together in infected seed samples. Quantitative PCR data showed that a positive relationship exists between the amount of *M. nivale* var. *nivale* and var. *majus* inoculum within seed samples, whereas no obvious trend was evident between the amount of *Fusarium* spp. and *M. nivale* var. *nivale* and var. *majus*. Differences in the ratio of the two sub-species between Scottish and English seed were evident though the predominance of the individual sub-species varied between years. Variety *majus* was generally present in greater proportions than var. *nivale* in Scotland and reference to previous studies suggests that this may be a result of increased stem-base infections by var. *majus* which may also be favoured by the presence of *T. acuformis*. The influence of climatic conditions such as

temperature, rainfall and leaf wetness on ear infections caused by var. *nivale* and *majus* and subsequent seed-borne contamination of wheat seed would provide interesting further work. Investigations aimed at determining the precise interaction between stem-base complex fungi and the two *M. nivale* sub-species and the effect of this on the contamination of wheat seed by var. *nivale* and var. *majus* would also appear to be an area requiring further work.

CHAPTER 6

STUDIES ON THE PATHOGENICITY OF ISOLATES OF *M. NIVALE* VAR. *NIVALE* AND VAR. *MAJUS* AS SEEDLING BLIGHT PATHOGENS

INTRODUCTION

Several fungal pathogens are able to cause Fusarium seedling blight under favourable environmental conditions. The relative pathogenicity of the three main seed-borne *Fusarium* species (*F. avenaceum*, *F. culmorum* and *F. graminearum*) and *M. nivale* was reviewed by Colhoun (1970). He considered *F. avenaceum* to be only weakly pathogenic towards wheat seedlings whereas *M. nivale*, *F. culmorum* and *F. graminearum* could cause significant disease though required different environmental conditions. Colhoun (1970) recorded large differences in pathogenicity between isolates of the pathogens used which were most variable within *M. nivale* though the author eliminated the existence of physiological races within *M. nivale* but did not state the basis for this conclusion. In similar experiments, Perry and Al-Hashimi (1983) reported large differences in the severity of seedling blight symptoms caused by *M. nivale* on barley seedlings.

Maurin (1993) reported that *M. nivale* var. *nivale* was more pathogenic towards wheat leaves than *M. nivale* var. *majus* though the author did not state how the experiments were performed. In contrast, Diamond and Cooke (1998) reported that isolates of var. *majus* were more aggressive towards detached wheat leaves than isolates of var. *nivale*.

Simpson *et al.* (2000) reported that *M. nivale* var. *nivale* caused more severe stem-base disease symptoms than *M. nivale* var. *majus* in wheat, rye and oats. Quantitative PCR assays, however, detected more *M. nivale* var. *majus* in inoculated wheat and rye plants than those inoculated with *M. nivale* var. *nivale* whereas the opposite effect was recorded for inoculated oats. The authors did not, however, investigate the pathogenicity of the two *M. nivale* sub-species as Fusarium seedling blight pathogens or state at what growth stage the seedlings used were inoculated.

The objectives of the work presented are to determine the pathogenicity of isolates of *M. nivale* var. *nivale* and var. *majus* as seedling blight pathogens from (i) surface borne inoculum (ii) naturally infected seed.

MATERIALS AND METHODS

EFFECT OF SPORE LOAD ON SEEDLING EMERGENCE AND THE SEVERITY OF SEEDLING BLIGHT DISEASE SYMPTOMS

Preparation of growth medium, pathogen inoculum and sowing of seed

John Innes No.2 potting compost was used. Stones and large pieces of organic matter were removed using a 0.5 mm sieve. Compost was autoclaved at 121°C for one hour and allowed to cool completely, the process was repeated twice. The compost was placed in plastic plant pots 7.5 x 7.5 cm to within 2.5 cm of the top of each pot.

Wheat seed cv. Cadenza was surface sterilised with a sodium hypochlorite solution (1.2 % available chlorine) containing 0.05% Tween 20 (Sigma) prior to inoculation. Spore suspensions of *M. nivale* var. *majus* (isolates SO30M and SO47M) and *M. nivale* var. *nivale* (isolate +315/22N and Nr4/N) were made in SDW (Chapter 2 page 36) to concentrations of $1x10^3$, $1x10^4$, $5x10^4$ and $1x10^5$ spores per ml (details of the isolates used is given in Appendix 2). The spore suspensions (2.5 ml) were mixed with 100 seeds for 3 min in a Petri-dish. Nine seeds were placed evenly in each pot and covered with 2 cm of sterile compost. Six replicate pots were used for each treatment.

Pots were placed in a controlled environment growth chamber and were arranged according to a randomised block design. The environmental conditions included a 12 hr Photoperiod (6800 lux), 80 % relative humidity, 8°C.

Assessment of disease

After six weeks, the number of seedlings present were recorded and sampled. The roots, remainder of the seed and leaves were removed and the lower 4 cm were assessed for disease severity and a disease index produced according to the methods described earlier (Chapter 2 page 38).

PATHOGENICITY OF *M. NIVALE* VAR. *NIVALE* AND VAR. *MAJUS* USING ARTIFICIALLY INOCULATED AND NATURALLY INFECTED SEED

Isolates used, inoculation of seed and production of naturally infected seed

Eight isolates of *M. nivale* var. *nivale* and eight isolates of *M. nivale* var. *majus* (Table 6.1) were used to artificially inoculate seed at a rate of $1x10^5$ spores per ml. The production of spore suspensions and inoculation of seed as described in Chapter 2 (pages 36 and 37). Seed infected with the same isolates of *M. nivale* var. *nivale* or var. *majus* was produced according to the methods described in Chapter 2 (page 37).

Table 6.1. Isolates of *M. nivale* var. *nivale* and var. *majus* used in artificial inoculation and natural infection experiments (further details of the isolates used is given in Appendix 2).

var. *nivale* isolates	var. *majus* isolates
Nr4/N	NL139M
SO28N	SO47M
SO47N	SO30M
SO04N	24/3/M
117/2/N	4/12/M
74/1/N	53/2/M
139/2/N	30/3/M
18/1/N	47/2/M

Extraction of DNA and quantification of M. nivale DNA

DNA was extracted from a 10 g sample of the grain obtained from each isolate according to the method described in Chapter 2 (page 40). The amount of *M. nivale* in each seed sample was quantified by competitive PCR using the JBM primers and JBMIS internal standard specific for *M. nivale* (Chapter 3, page 69). Diagnostic PCR's using *M. nivale* var. *nivale* and var. *majus* specific primers (Nicholson *et al.*, 1996a) were also

performed as a test for cross-contamination of the seed during inoculation. PCR reaction conditions were as described in Chapter 2 (page 41) for diagnostic PCR.

Comparison between sub-species

Sowing of seed and disease assessments

The preparation of compost and inoculum, sowing of seed, growing conditions and assessment of seedling blight disease symptoms were as described in the previous experiment with the exception that no spore suspension was applied to seed which was naturally infected. Data was analysed by ANOVA with sub-species and isolate as variates.

RESULTS

EFFECT OF SPORE LOAD ON SEEDLING EMERGENCE AND THE SEVERITY OF STEM BASE DISEASE SYMPTOMS

No significant ($P > 0.05$) difference was observed between the emergence of seed inoculated with spores of either *M. nivale* var. *nivale* or var. *majus* at the three lowest rates used. Overall, no significant ($P > 0.05$) difference between the number of seedlings which emerged from seed inoculated with *M. nivale* var. *nivale* and those inoculated with *M. nivale* var. *majus* were observed.

Stem base disease symptoms of Fusarium seedling blight infection were observed on emerged seedlings from seed artificially inoculated with either *M. nivale* var. *nivale* or var. *majus* at all four spore loads. The severity of symptoms for all four isolates increased with increasing spore load (Figure 6.1). The severity of stem-base symptoms (disease index) was greatest for the two isolates of *M. nivale* var. *majus* than for the two isolates of *M. nivale* var. *nivale*, the difference being greatest and most significant ($P < 0.05$) at the highest spore load (1×10^5 spores per ml).

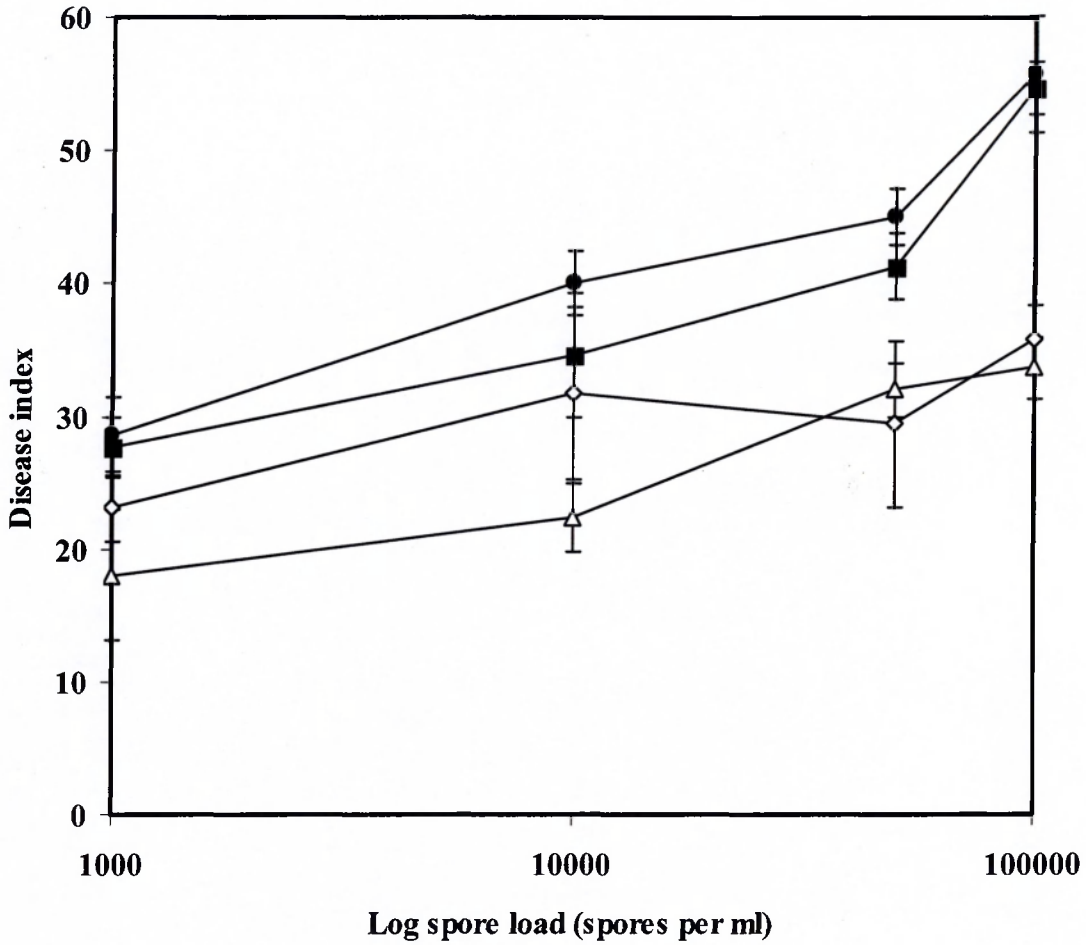


Figure 6.1. The relationship between spore load used to artificially inoculate wheat seed (cv. Cadenza) at concentrations of $1x10^3$, $1x10^4$, $5x10^4$ and $1x10^6$ and disease index observed on seedlings at growth stage 12 for isolates of *M. nivale* var. *majus* (●) isolate SO30M, (■) isolate SO47M, and *M. nivale* var. *nivale* : (◇) isolate +315/22N, (△) isolate Nr4/N. Bars indicate standard error of the means Control = 2.125.

PATHOGENICITY OF *M. NIVALE* VAR. *NIVALE* AND VAR. *MAJUS* USING ARTIFICIALLY INOCULATED AND NATURALLY INFECTED SEED

Emergence of seedlings from seed artificially inoculated with spores of *M. nivale* var. *majus* was significantly lower than the emergence from untreated seed or from seed artificially inoculated with spores of *M. nivale* var. *nivale* (Figure 6.2).

Seedling emergence from artificially inoculated seed was significantly ($P < 0.05$) reduced compared to untreated seed for *M. nivale* var. *majus* isolates 24/3/M, 53/2/M,

30/3/M and 47/2/M. Emergence was not reduced significantly ($P > 0.05$) compared to untreated seed for any seed inoculated with any isolates of *M. nivale* var. *nivale*.

Stem-base disease severity on emerged seedlings was significantly greater on seedlings produced from *M. nivale* var. *majus* or var. *nivale* inoculated seed than from untreated seed. Seed artificially inoculated with spores of *M. nivale* var. *majus* produced seedlings with significantly more severe symptoms than from those inoculated with *M. nivale* var. *nivale* (Figure 6.3).

All isolates of *M. nivale* var. *majus* caused stem-base disease symptoms the severity of which was significantly ($P < 0.05$) greater than those on seedlings produced from untreated seed (Figure 6.3). All isolates of *M. nivale* var. *nivale* with the exception of isolate Nr4/N produced seedlings with significantly more severe stem-base symptoms than on those produced from untreated seed. Significant differences in the severity of stem-base symptoms between isolates of both sub-species were also observed; var. *majus* isolate SO30M produced seedlings with significantly ($P < 0.05$) less severe symptoms than isolates 53/2/M, SO47M and 4/12/M. Isolate Nr4/N produced seedlings with significantly ($P < 0.05$) less severe symptoms than any other var. *nivale* isolate. Isolate 117/2/N produced seedlings with significantly ($P < 0.05$) less severe symptoms than from isolates 139/2/N, SO04/N and SO47/N. Isolates 74/1/N and SO28N produced seedlings with significantly ($P < 0.05$) less severe symptoms than SO47/N.

Diagnostic PCR using *M. nivale* var. *nivale* and var. *majus* specific primers sets showed that no cross-contamination of inoculum between sub-species had occurred. *Microdochium nivale* was detected and quantified in all seed batches produced under glasshouse conditions. The amount of *M. nivale* DNA quantified for seed from each seed of the 16 inoculated seed batches is presented in Table 6.2.

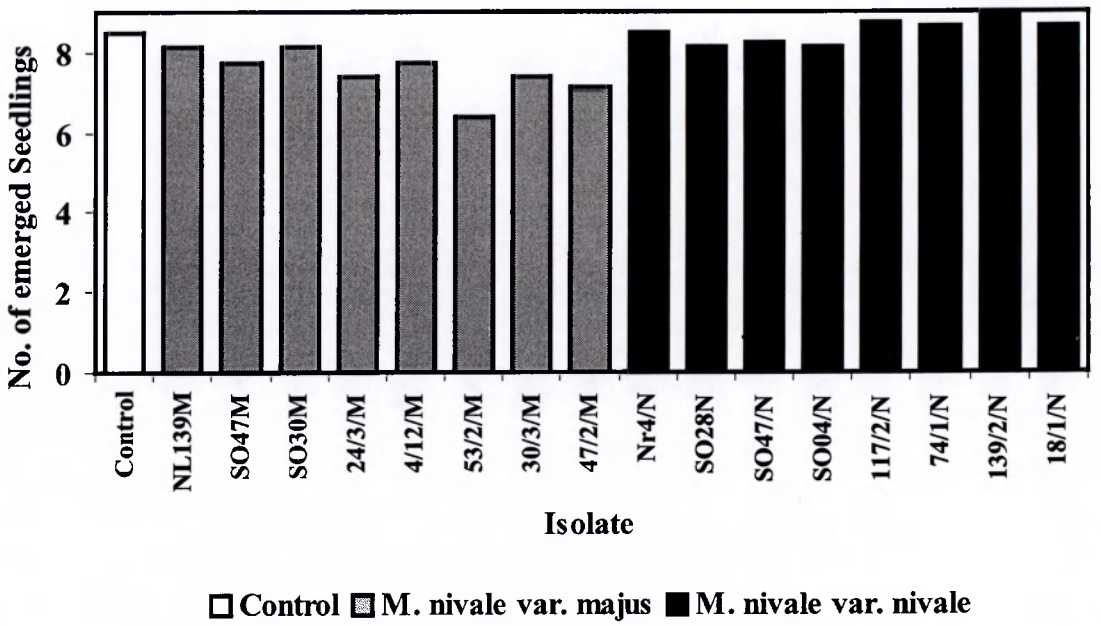


Figure 6.2. Effect on seedling emergence of spores of eight isolates of *M. nivale* var. *majus* and eight isolates of *M. nivale* var. *nivale* applied to wheat seed cv. Cadenza (at a concentration of 1 x 10^5 spores ml^{-1}) compared to a water control (SEM = 0.446; (LSD (*P* = 0.05) = 0.883). D of F = 135; CV = 11.1%.

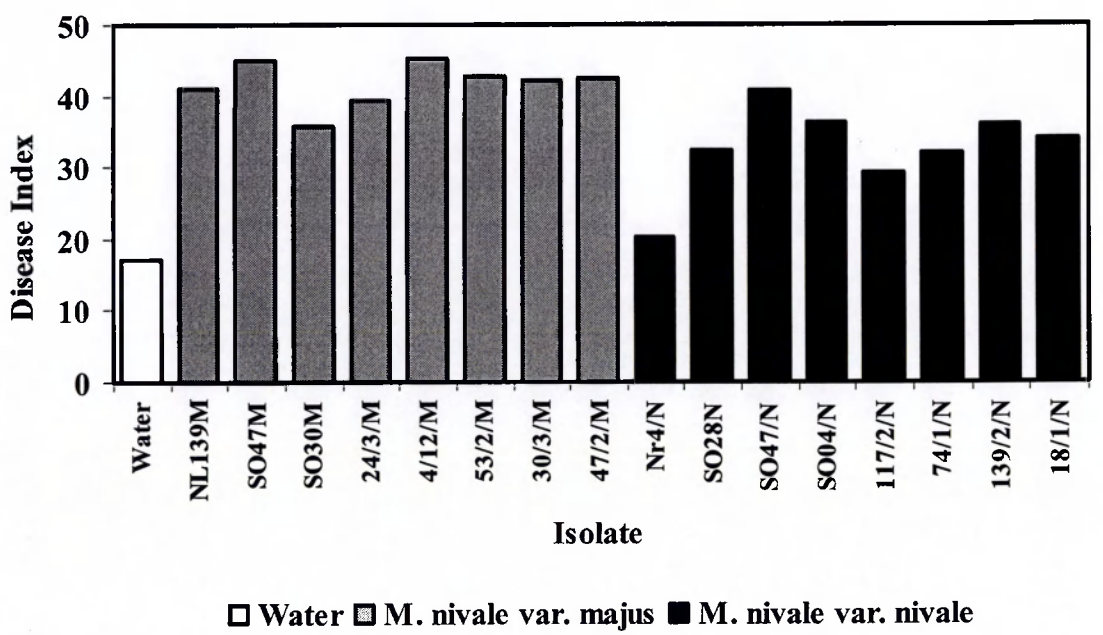


Figure 6.3. Effect on seedling blight disease severity on emerged seedlings of spores of eight isolates of *M. nivale* var. *majus* and eight isolates of *M. nivale* var. *nivale* applied to wheat seed cv. Cadenza (at a concentration of 1 x 10^5 spores ml^{-1}) compared to a water control (SEM = 3.38; (LSD (*P* = 0.05) = 6.69). D of F = 135; CV = 18.8%.

Table 6.2. Isolates of *M. nivale* var. *nivale* and var. *majus* used to infect wheat plants cv. Cadenza at anthesis in the glasshouse and the amount of *M. nivale* DNA quantified in the resultant seed.

***M. nivale* var. *majus* isolate used**	***M. nivale* DNA (pg ng^{-1} total DNA)**	***M. nivale* var. *nivale* isolate used**	***M. nivale* DNA (pg ng^{-1} total DNA)**
NL139/M	0.86	Nr4/N	0.27
SO47/M	1.33	SO28N	0.53
SO30/M	0.73	SO47/N	1.15
24/3/M	0.95	SO04/N	0.22
4/12/M	0.93	117/2/N	1.12
53/2/M	0.62	74/1/N	0.94
30/3/M	1.14	139/2/N	1.22
47/2/M	0.95	18/1/N	0.85

Control = 0

Overall, seedling emergence from naturally infected seed was significantly ($P < 0.05$) lower for seed produced from *M. nivale* var. *majus* infected plants than from untreated or *M. nivale* var. *nivale* infected seed across the isolates used (Figure 6.4). In addition, overall, no significant difference in seedling emergence was observed between *M. nivale* var. *nivale* and untreated seed. All *M. nivale* var. *majus* isolates and var. *nivale* isolates SO28N, SO47N and SO04N reduced emergence significantly ($P < 0.05$) compared to the untreated control. The emergence of seedlings from seed produced from plants inoculated with spores of *M. nivale* var. *majus* isolates SO30M, 4/12/M and 30/3/M was significantly greater than from seed produced from plants inoculated with spores of NL139M or SO47M. The emergence of seedlings from seed produced from plants inoculated with spores of var. *nivale* isolate Nr4/N was significantly ($P < 0.05$) greater than that for seed produced from plants inoculated with isolates SO28N, SO47N or

SO04N. Emergence from 18/1/N infected seed was significantly ($P < 0.05$) greater than from SO47N infected seed.

The severity of stem-base symptoms on emerged seedlings was significantly greater on seedlings produced from *M. nivale* var. *majus* infected seed than from var. *nivale* infected seed or from untreated seed (Figure 6.5). Seed infected with var. *nivale* produced seedlings with significantly ($P < 0.05$) more severe stem-base symptoms than from untreated seed. All isolates used produced seedlings with more severe stem-base symptoms than the untreated seed with the exception of isolate SO04N. Of the *M. nivale* var. *majus* isolates used, isolate SO47M produced seedlings with significantly more severe symptoms than all other var. *majus* isolates (Figure 6.5).

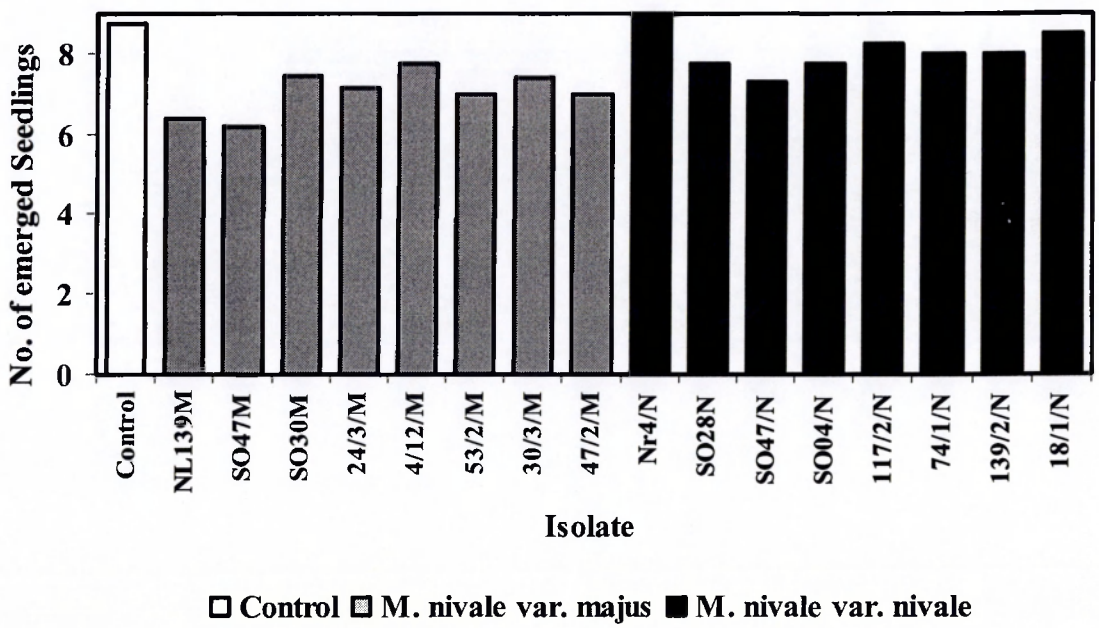


Figure 6.4. Effect of seed-borne inoculum of eight isolates of *M. nivale* var. *majus* and eight isolates of *M. nivale* var. *nivale* on the emergence of wheat seed cv. Cadenza compared to a water conrol (SEM = 0.502; (LSD ($P = 0.05$) = 0.994). D of F = 135; CV = 13.2%.

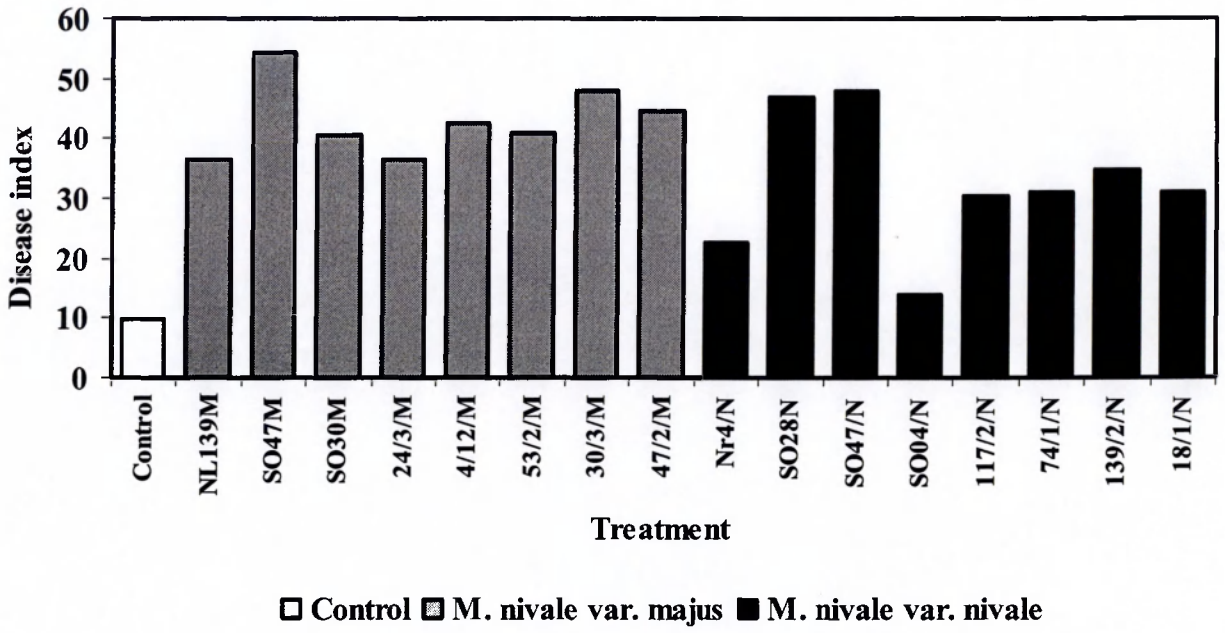


Figure 6.5. Effect of seed-borne of eight isolates of *M. nivale* var. *majus* and eight isolates of *M. nivale* var. *nivale* on the severity of seedling blight disease symptoms (disease index) on emerged wheat seedlings cv. Cadenza compared to a water control (SEM = 4.25; (LSD (P = 0.05) = 8.42). D of F = 135; CV = 23.6%.

Seed infected with isolate 30/3/M produced seedlings with more severe stem-base symptoms than isolates NL139M and 24/3/M. Seedlings produced from *M. nivale* var. *nivale* isolate Nr4/N infected seed produced seedlings with less severe symptoms than seedlings produced from isolates SO28N, SO47N, 139/2/N or 18/1/N infected seed. Seed infected with isolates 74/1/N 139/2/N or 18/1/N produced seedlings with significantly less severe stem-base symptoms than those produced from SO28N or SO47/N.

Relationship between seedling blight symptoms from artificially inoculated and naturally infected seed

A significant (P = 0.009) relationship between the emergence of seedlings from artificially inoculated and from naturally infected seed was observed for the isolates of *M. nivale* used (Figure 6.6). Three of the eight *M. nivale* var. *majus* isolates and six of the seven var. *nivale* isolates tested were above the regression line obtained (Figure 6.6). A significant (P = 0.016) relationship between stem-base disease severity on emerged

seedlings from artificially inoculated and naturally infected seed was observed. Seven of the eight *M. nivale* var. *majus* isolates and one *M. nivale* var. *nivale* isolate were above the regression line (Figure 6.7a).

Relationship between* M. nivale *DNA and emergence and disease severity

The relationship between the number of emerged seedlings from naturally infected seed and the amount of *M. nivale* DNA quantified in the seed was not significant (P = 0.151). The relationship between disease severity and the amount of *M. nivale* DNA was significant (P = 0.010) (Figure 6.7b).

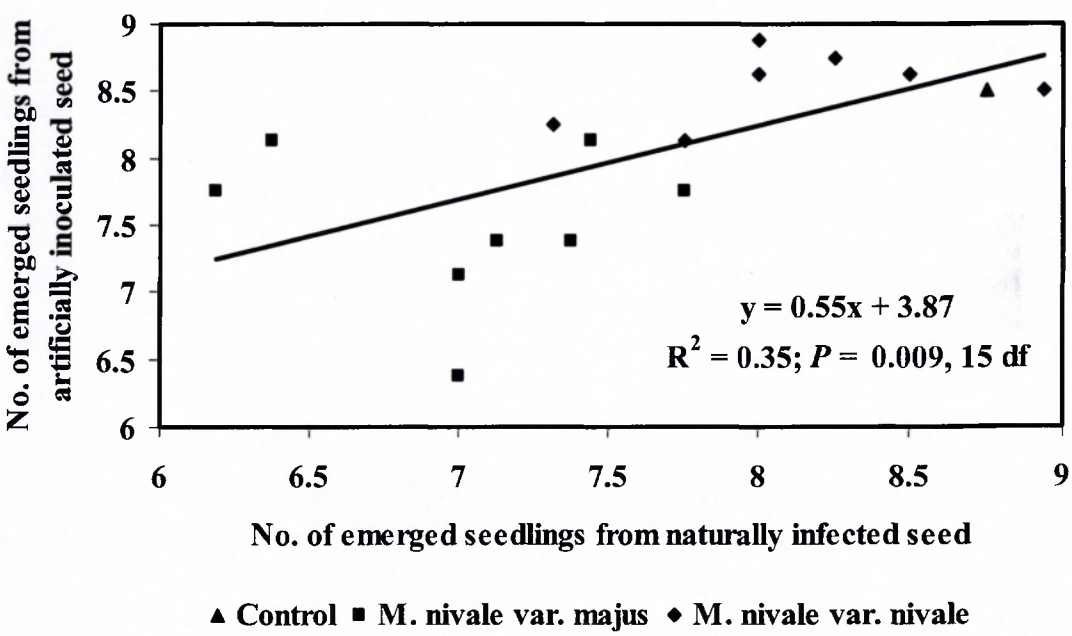


Figure 6.6. Relationship between the number of seedlings that emerged from wheat seed cv. Cadenza artificially inoculated with spores of individual isolates of *M. nivale* var. *nivale* or var. *majus* (y axis) against the number of seedlings that emerged from seed naturally infected with the same isolates of *M. nivale* var. *nivale* or var. *majus* (x axis).

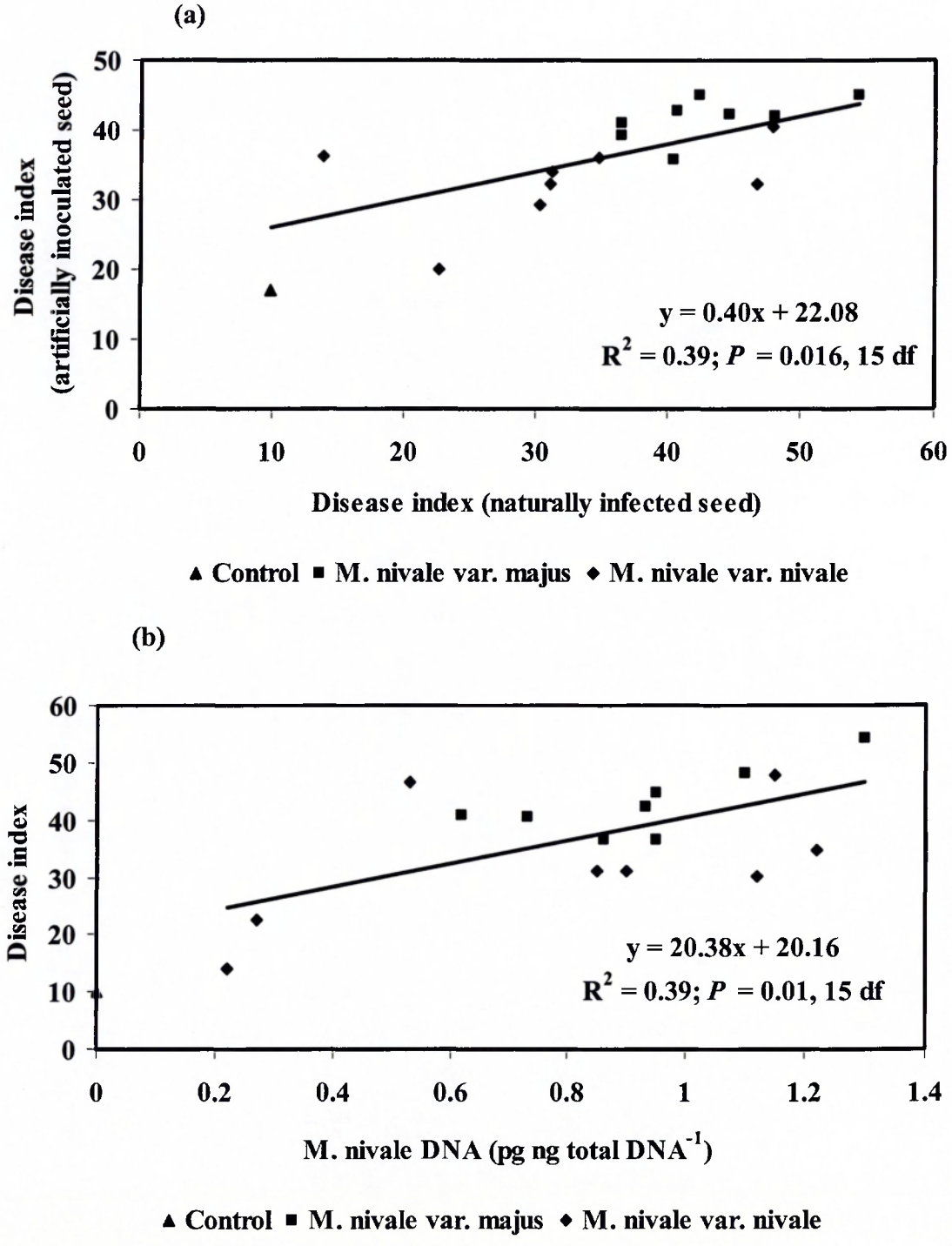


Figure 6.7. (a) Relationship between the severity of seedling blight disease symptoms on emerged seedlings (disease index) observed from wheat seed cv. Cadenza artificially inoculated with spores of individual isolates of *M. nivale* var. *nivale* or var. *majus* (y axis) against seedling blight disease symptoms from seed naturally infected with the same isolates of *M. nivale* var. *nivale* or var. *majus* (x axis) (b) Relationship between the amount of *M. nivale* DNA quantified in seed cv. Cadenza using JBM quantitative PCR assay and seedling blight disease severity (disease index) observed on the resultant seedlings.

DISCUSSION

Previous experiments within this study have shown a clear relationship between the amount of *M. nivale* inoculum in seed samples and symptoms of seedling blight (emergence and stem-base disease severity) (Chapter 4). Differences in pathogenicity between var. *majus* and var. *nivale* were observed. However, as most of the seed samples used contained inoculum of both of the *M. nivale* sub-species, conclusions on the relative pathogenicity of the two were confounded. Furthermore, the quantification of *M. nivale* within a bulk seed sample means that inoculum present on the seed surface and within the seed coat is detected using quantitative PCR. The relative importance of surface-borne inoculum and that which is present within the seed is difficult to determine. The experiments in this study have shown that inoculum of *M. nivale* var. *majus* applied as spores at a high enough concentration to the surface of wheat seeds causes reductions in emergence. No such reductions in emergence were evident for *M. nivale* var. *nivale* which further supports the findings of the previous investigation (Chapter 4) that var. *majus* inoculum was most closely related to reduced emergence than var. *nivale.* A positive relationship between the amount of *M. nivale* inoculum present on the surface of wheat seeds and the severity of stem-base seedling blight symptoms was observed. Experiments performed in Chapter 4 showed a significant relationship between the amount of *M. nivale* inoculum detected using quantitative PCR assays or plate counts and reductions in emergence and the severity of stem-base disease symptoms exists. This study has shown that the same is true for inoculum present on the seed surface in the form of fungal spores. Millar and Colhoun (1969) reported that the severity of seedling blight symptoms increased with increasing spore loads of one isolate of *M. nivale* applied to the surface of winter wheat seed cv. Astle up to 50,000 spores per seed. When they applied spores at rate of 500,000 spores per seed the severity of stem-base symptoms was reduced slightly compared to 50,000. The authors produced a seedling blight severity index which included seedlings which did not emerge making the effects of the inoculum load on the individual

disease phases from their experiments impossible to determine. Hare (1997) reported reductions in emergence caused by isolates of *M. nivale* var. *majus* applied as spores to the surface of winter wheat seeds cv. Brigadier at a rate of 4×10^3 spores per seed. Isolates of *M. nivale* var. *nivale* did not reduced emergence significantly compared to the untreated. Isolates of both *M. nivale* sub-species caused stem-base disease symptoms the severity of which was greatest for the isolates of *M. nivale* var. *majus* that he used. Interestingly, Hare (1997) reported large variations in emergence and the severity of stem-base disease severity between var. *majus* isolates used, such variation was less evident for the isolates of *M. nivale* var. *nivale* that he used. The results from the present study showed generally greater variability in seedling blight disease symptoms from naturally infected compared to artificially inoculated seed experiments

The production of infected grain under glasshouse conditions allows the pathogenicity of individual isolates to be investigated; this is often not possible using grain produced under field conditions where other seedling blight pathogens may contaminate seed samples. As with surface applied inoculum, glasshouse produced seed produced seedlings with symptoms of seedling blight infection from both *M. nivale* var. *nivale* and var. *majus*. The relationship between emergence from artificially inoculated and naturally infected seed and the severity of stem-base disease symptoms from the two methods of inoculation was significant, though the variation accounted for was low due to a high degree of variability. The severity of stem-base symptoms was more variable for isolates of var. *nivale* than var. *majus*. Other evidence for increased variability within the var. *nivale* sub-species compared to the more homogenous var. *majus* sub-species was reported by Parry *et al.*, (1995b) as well as suggestions of slightly increased genetic variability within the EF-1α gene earlier in this study (Chapter 3). Parry *et al.* (1995) suggested that the ability of var. *nivale* to reproduce heterothallically in nature may lead to increased variability compared to the less variable var. *majus* which reproduces homothallically in nature. Thus the variability in pathogenicity between the var. *nivale* isolates tested in this

experiment may be associated with the genetic variability of the var. *nivale* sub-species. Initially, Maurin *et al.* (1993) suggested that isolates of *M. nivale* var. *nivale* were more pathogenic towards wheat plants than var. *majus*. However, in a further experiment, Maurin *et al.* (1995) reported no clear correlation between *M. nivale* variety and pathogenicity towards wheat seedlings. The differential host response reported by Simpson *et al.* (2000) and conflicting conclusions of Maurin *et al.* (1993 and 1995) serve to further highlight the variable nature of *M. nivale*. Taken together, the conclusions of the previous workers and the results from earlier investigations within this study provide strong evidence that variety *majus* is indeed more pathogenic towards wheat than variety *nivale*. As no cross-contamination between the two sub-species in the production of the naturally infected grain was observed in this experiment, it is assumed that no cross contamination between isolates of the same sub-species occurred. The use of molecular markers such as RAPD's, AFLP's or Simple Sequence Repeats (SSR's) which were identified for specific isolates of *M. nivale* would allow confirmation that no contamination had occurred. The use of such molecular markers would also provide a means of detecting specific isolates of each sub-species in infected plant material throughout the growing season. Further investigations, therefore, could focus on the development and application of these techniques to specific isolates of *M. nivale*. This would allow the relationship between the findings of Simpson *et al.* (2000), Maurin *et al.* (1993 and 1995) and those of the present investigation and the pathogenicity of *M. nivale* var. *nivale* and var. *majus* under field conditions to be determined.

CHAPTER 7

STUDIES ON FUNGICIDE SEED TREATMENT EFFICACY FOR THE CONTROL OF FUSARIUM SEEDLING BLIGHT OF WHEAT USING PCR

INTRODUCTION

The primary method employed for the control of Fusarium seedling blight in the UK is the use of seed treatments. Several seed treatments are available in the UK for the control of Fusarium seedling blight (Chapter 1). Seed-borne *M. nivale* was only partially controlled by organomercury as the chemical did not effectively eradicate deep-seated infections, whereas triazole treatments provide only partial control and are formulated with benzimidazole fungicides which are now largely ineffective against *M. nivale* due to the development of resistance (Koch *et al.*, 1992). The introduction of the phenylpyrrole fungicides fenpiclonil and fludioxonil as seed treatments for the control of Fusarium seedling blight represented a previously un-used family of fungicide chemistry. As with any treatment for the control Fusarium seedling blight, the efficacy of seed treatments towards the individual causal agents of Fusarium seedling blight is difficult to determine under field conditions. Visual disease symptoms cannot be used as several pathogens can cause the same symptoms. The *in vitro* sensitivity of fungal pathogens towards fungicides can give an indication of the likely performance towards a particular fungal species prior to expensive field trials being performed. Experiments performed under controlled environmental conditions allow comparisons to be made between the control achieved by different seed treatments under different specific conditions. Since the confirmation by Nicholson *et al.* (1996a) of the existence of two sub-species within *M. nivale* as earlier suggested by Lees *et al.* (1995), no results have been reported on the efficacy of fungicides used to control the individual *M. nivale* sub-species.

The objectives of this Chapter were (i) to determine the *in vitro* efficacy of fungicide seed treatments towards isolates of *M. nivale* var. *nivale* and *M. nivale* var. *majus* (ii) to study the effect of temperature on the performance of seed treatments towards seedling blight caused by *M. nivale* var. *nivale* and *M. nivale* var. *majus* (iii) to study the

field performance of seed treatments towards seedling blight of wheat caused by *M. nivale* var. *nivale*, *M. nivale* var. *majus* and *Fusarium* spp.

MATERIALS AND METHODS

IN VITRO SENSITIVITY OF ISOLATES OF *M. NIVALE* VAR. *NIVALE* AND *M. NIVALE* VAR. *MAJUS* TOWARDS SIX FUNGICIDE SEED TREATMENTS

Isolates used and fungicide concentrations

Ten single spore isolates of both *M. nivale* var *nivale* and *M. nivale* var. *majus* were used (Chapter 2, page 36 and Appendix 2). A 5 mm mycelial plug was taken from the edge of fungal colonies (for each of the 20 isolates) and placed centrally onto PDA plates amended with one of six fungicides seed treatments (Table 7.1). PDA without fungicide was used as a control and four replicates of each treatment set up. Plates were incubated in the dark at 19°C for six days and colony diameters were measured in two directions (one at 90° to the other). The mean diameter was calculated and used for the prediction of the effective concentration required to reduce fungal growth by 50% (EC_{50}).

A preliminary experiment established a range of chemical concentrations to use for the determination of EC_{50}'s. An approximate EC_{50} estimate was made around which six rates were chosen based on the concentration of active ingredient(s). When a product contained more than one active ingredient, the rate was based on the combined total of active ingredients; concentrations used are presented in Table 7.1. Due to the differing activity of each seed treatment, the range of concentrations used for each product differed.

Statistical analysis

EC_{50} estimates were made for each *M. nivale* var. *nivale* and var. *majus* isolate towards each fungicide seed treatments by probit analysis (Genstat) using the mean colony diameter for all replicates at each concentration. Comparisons between fungicide sensitivity between each sub-species for each fungicide were made by ANOVA. EC_{50} data

was power transformed using constant as 0 and m function as –0.5 to obtain normal distributions.

Table 7.1. Rates of chemical used for EC_{50} determination (based on active ingredient(s)), concentrations chosen were determined from a preliminary experiment.

Anchor (Carboxin + Thiram) (mg l^{-1})	Baytan (Carbendazim+ Triadimenol) (mg l^{-1})	Beret Gold (Fludioxonil) (mg l^{-1})	Panoctine (Guatazine + Imazalil) (mg l^{-1})	Sibutol (Bitertanol + fuberidazole) (mg l^{-1})	A8179b (Difenoconazole) (mg l^{-1})
1.0	0.5	0.01	0.15	0.05	0.05
5.0	2.0	0.02	0.5	0.1	0.1
10.0	5.0	0.04	2.0	0.2	0.2
15.0	10.0	0.05	5.0	0.3	0.4
25.0	20.0	0.06	10.0	0.4	0.6
60.0	36.0	0.08	20.0	0.5	0.8

EFFECTS OF TEMPERATURE ON THE PERFORMANCE OF THREE FUNGICIDE SEED TREATMENTS TOWARDS SEEDLING BLIGHT OF WHEAT CAUSED BY SEED-BORNE *M. NIVALE* VAR. *NIVALE* AND VAR. *MAJUS*

Seed lots used

Seed lots of winter wheat cv. Cadenza infected with isolates of *M. nivale* var. *nivale* or var. *majus* were produced according to the method described in Chapter 2 (page 37). Twelve plants were inoculated at GS65 with spores of either *M. nivale* var. *nivale* (isolates: 139/2/N, 117/1/N, 74/1/N and 42N) or *M. nivale* var. *majus* (Isolates: 47/2/M, 24/3/M, 44M and 30/3/M). Two lots of plants were used for each sub-species and inoculated with spores at concentrations of 5×10^4 spores per ml and 2×10^5 spores per ml to produce two seed lots infected with each sub-species. Spores were applied at a rate of 2 ml of spore suspension per ear using a hand held sprayer. Grain was harvested at GS92, DNA extracted according to the method described in Chapter 2 (page 40) and *M. nivale* quantified using the quantitative PCR assay described in Chapter 3 (page 69). Diagnostic

PCR using primers described by Nicholson *et al.* (1996a) and the JBF primers (Chapter 3, page 69) were used to determine if any cross-contamination of inoculum had occurred

Application of seed treatments

Seed treatments used were Sibutol (Bitertanol + Fuberidazole 375 +23g l), Beret Gold (fludioxonil 25g l^{-1}) and Celest Extra (Difenoconazole + fludioxonil 25 + 25g l^{-1}). Seed treatments were applied at standard field rates of 1.5 l tonne^{-1} of seed, 2.0 l tonne^{-1} of seed and 2.0l tonne^{-1} of seed respectively. Seed (20 g) was placed in a conical flask and agitated for 3 min using a Griffin flask shaker (Griffin and George Ltd, UK) seed treatments were diluted two fold in SDW and applied to each seed sample separately using a syringe, water was used as a control.

Effect of temperature on fungicide efficacy

Growth medium, sowing of seed and experimental conditions

John Innes No.2 potting compost was used, stones and large pieces of organic matter were removed from the compost using a 0.5 mm sieve. Compost was autoclaved at 121°C for one hour and allowed to cool for 16 h, the process was repeated twice. Compost was placed in plastic plant pots 7 x 7 x 8 cm to within 2.5 cm of the top. Nine seeds were placed in each pot, compost was placed on the surface up to the rim of the pot and firmed by hand. Pots were placed in a growth chamber (Conviron, Canada) and were arranged according to a randomised block design, with eight replicates.

Experiments were performed at temperatures of 16, 12 and 8°C, a Photoperiod of 12 hr and 80 % relative humidity. A further experiment was performed at 4°C for which nine seeds were placed in 2.5 cm of compost in a glass jar (11.5 (height) x 6 cm (diameter)). Jars were placed in an illuminated, refrigerated incubator according to a randomized block design with eight replicates.

At GS12, seedlings were sampled. Roots, the remainder of the seed and leaves were removed and the lower 4 cm of stem-base were assessed for disease (Chapter 2, page

38). The number of emerged seedlings from each, and the number seedlings showing stem-base symptoms was recorded. Seedlings from each treatment for each experiment were freeze-dried prior to DNA extraction. DNA was extracted (Chapter 2, page 39) and diagnostic PCR used to determine whether any cross-contamination had occurred between treatments.

Statistical analysis

Data were analysed using a two-way ANOVA (Genstat) with temperature (4, 8, 12 and 16°C) and treatment (untreated, Sibutol, Beret Gold and Celest Extra) as factors. Symptom incidence data was Logit transformed to obtain normal distributions.

FIELD PERFORMANCE OF FUNGICIDE SEED TREATMENTS

Source of seed

Standard crop husbandry practices were used to maintain 12 plots (10 x 2 m) of wheat cv. Hussar in the 1997/98 growing season and cv. Equinox in the 1998/99 growing season. Four plots were inoculated at mid-anthesis (GS65) separately with a conidial suspension from five isolates (2×10^5 spores ml^{-1}) of seedling blight pathogens at a rate of 33 ml/m^2 using a knapsack sprayer. In 1998, plots were inoculated with *F. culmorum* (seed lot 2), *F. graminearum* (seed lot 3) or *M. nivale* var. *majus* (seed lot 4). In 1999, plots were inoculated with *F. culmorum* (seed lot 2), *M. nivale* var. *majus* (seed lot 3) or *M. nivale* var. *nivale* (seed lot 4).

Following inoculation, overhead mist irrigation (Access irrigation, Northampton, UK) was applied in both years to plots for a period of 3 min every 30 min between 08.00 and 18.00 h (B.S.T.) for 21 days to provide conditions conducive to ear infection. Four uninoculated, non-misted plots were used as guards between each set of inoculated misted plots. Grain was harvested at GS92 using a Seedmaster Plot combine (Wintersteiger,

Austria). In each year, a sample of commercial grain of the same variety was obtained for use in seed treatment trials (seed lot 1).

Grain analysis

The percentage of seeds infected with *M. nivale* and *Fusarium* spp. was determined for the four seed lots used in each year using the agar plate count methods (Chapter 2, page 35). For grain used in 1999, the incidence of *M. nivale* var. *nivale* and var. *majus* was also determined for the four seed lots. Colonies of *M. nivale* emanating from surface sterilised grain were removed to a fresh PDA plate and incubated at 19°C for one week under NUV lights (12 h Photoperiod). After one week, the DNA was extracted from a small amount of fungal mycelium according to the extraction method for fungal colonies Chapter 2 (page 38). The *M. nivale* sub-species was determined for each isolate by PCR amplification using the primers described by Nicholson *et al.* (1996a). PCR reaction conditions and PCR product analysis were as described in Chapter 2 (page 41) for diagnostic PCR.

DNA was extracted from four 10 g samples taken from each of the four seed batches used in each year (Chapter 2, page 40). The amount of *M. nivale* var. *nivale*, var. *majus* and *Fusarium* spp. was determined using quantitative PCR assays (Chapter 3).

FIELD TRIALS

Treatments and trial design

Three seed batches produced from inoculated field plots in 1998 and 1999 (seed lots 2-4) and the commercial (uninoculated) seed lot in each year (lot 1) were used in fungicide seed treatment efficacy trials. Seed was treated with either Beret Gold (a.i. fludioxonil: 24.3 g l^{-1}) (2l $tonne^{-1}$) or Sibutol (a.i. bitertanol + fuberidazole: 375 + 23 g l^{-1}) (1.5l $tonne^{-1}$). Untreated seed was used as a control and drilled at a rate of 275 m^2 in the 1998 trial and 350 m^2 in the 1999 trial according to a randomized block design with four replicates at Edgmond, Shropshire, UK.

Germination tests

Germination tests were performed on all untreated and treated seed lots on grain produced in both 1998 and 1999. One hundred seeds were placed on moist filter paper and incubated in the dark at 18°C or 4°C for one or two weeks respectively, four replicates were used. After incubation the number of germinated seeds was recorded. Data was analysed using ANOVA with seed lot (1-4) and treatment (untreated, Sibutol and Beret Gold) as factors.

Field trial assessments

Stand counts were made at GS12 by counting the number of emerged seedlings along either side of 0.5 m sections of rows within each plot, six observations were made in each plot. Thirty seedlings were removed from one half of each plot at GS12, the roots, remaining seed coat and any soil debris were removed before an assessment of severity of stem-base symptoms was made using the assessment key outlined in Chapter 2 (page 38). Seedlings were cut to 4 cm in length from the base of the stem, washed, placed in plastic kartell tubes (Fisher, UK) and freeze-dried prior to DNA extraction. In the 1999 trial, a further thirty seedlings were removed from the same side of each plot. The roots, remaining seed coat and any soil debris were removed before the seedlings were cut 1 cm in length from the base, surface sterilised in a 5 % bleach solution (0.6 % available chlorine) and placed on PDA (three per plate) amended with streptomycin sulphate (130 mg l^{-1}). Plates were incubated for one week under the same conditions after which colonies emanating from individual seedlings and conforming to the colony characteristics of *M. nivale* (production of salmon pink mycelium) were removed to a fresh PDA plate. Plates were incubated under the same conditions, the number of colonies which conformed to the colony characteristics of *Fusarium* spp. was recorded. DNA was extracted from all colonies of *M. nivale* according to the fungal colony extraction method (Chapter 2, page

38) and the *M. nivale* sub-species determined using the primers described by Nicholson *et al.* (1996a). PCR reaction conditions and PCR product analysis were as described in Chapter 2 (page 41) for diagnostic PCR.

A further stand count was made at GS25 using that side of the plot not examined at the previous assessment. The same assesssment method was used as at GS12; thirty seedlings were again removed from each plot. Seedlings were washed, the roots and remaining seed coat were then removed. Seedlings were chopped to 4 cm from the base and an assessment of disease severity made (Chapter 4). Seedlings from each plot were placed in a plastic kartell tube and freeze-dried prior to DNA extraction. For the 1999 trial, an assessment of stem-base disease severity was made at GS59; thirty plants were removed from each plot and the severity of stem-base symptoms observed was recorded (Chapter 2, page 38). The main stem from each plant was removed, cut from the base to 4 cm in length, placed in a kartell tube and freeze dried prior to DNA extraction. The number of tillers per plant and number of dead tillers per plant was also recorded.

DNA was extracted (Chapter 2, page 39) and diluted from seedling samples taken at GS12 and GS25 in 1998 and 1999 and also plant samples taken at GS59 in the 1999 trial according to methods described in Chapter 2 (page 39). The amount of *M. nivale* in samples from each plot for GS12 and GS25 in the 1998 and 1999 trials was determined using the quantitative PCR assays (Chapter 3, page 69). The amount of *Fusarium* spp. in samples from the 1998 and 1999 trials was determined using quantitative PCR assays (Chapter 3) which were performed three times. For the 1999 trial, the amount of *M. nivale* var. *nivale* and var. *majus* was determined in samples taken at GS12, 25 and 59 using quantitative PCR assay (Chapter 3, page 69). The ratio of *M. nivale* var. *nivale* to *M. nivale* var. *majus* was determined for each treatment. For samples taken at GS12, PCR reactions were repeated three times and for samples taken at GS25 and GS59, PCR reactions were performed twice.

Data analysis

Quantitative PCR data were Log transformed to obtain normal distributions then analysed initially by accumulated analysis of variance then by two-way analysis of variance with treatment (untreated, Sibutol and Beret Gold) and seed lot (1-4) as factors. Stand count and disease index data were analysed by two way ANOVA with block (1-4), seed lot (1-4) and treatment (untreated, Sibutol and Beret Gold) as factors.

RESULTS

IN VITRO SENSITIVITY OF *M. NIVALE* VAR. *NIVALE* AND *M. NIVALE* VAR. *MAJUS* TOWARDS SIX FUNGICIDE SEED TREATMENTS

EC_{50} values for Beret Gold were lowest of the fungicides used ranging from 0.028 to 0.075 mg l^{-1} for *M. nivale* var. *majus* and 0.037 to 0.067 mg l^{-1} for var. *nivale* (Figure 7.1a). EC_{50}'s for Sibutol ranged from 0.15 to 0.51 for var. *majus* and from 0.17 to 0.41 for var. *nivale* (Figure 7.1b). Sensitivity to Difenoconazole varied from 0.29 to 0.60 mg l^{-1} for var. *majus* and 0.36 to 0.58 mg l^{-1} for var. *nivale* (Figure 7.2a). EC_{50} values for Panoctine ranged from 0.47 to 1.29 mg l^{-1} for var. *nivale* and for var. *majus* from 0.69 to 1.48 mg l^{-1} (Figure 7.2b). No EC_{50} estimate for panoctine for isolate SO04N was made as this isolate showed growth of less than 50% of the untreated at the lowest fungicide concentration used, and zero growth at all other concentrations. For Anchor, EC_{50}'s ranged from 6.22 to 22.82 mg l^{-1} for *M. nivale* var. *majus* and 8.26 to 25.36 mg l^{-1} for var. *nivale* (Figure 7.3a). Sensitivity to Baytan ranged from 1.30 to 12.21 for *M. nivale* var. *nivale* and from 1.26 to 14.00 for var. *majus* (Figure 7.3b).

Two-way ANOVA showed a highly significant ($P < 0.001$) difference in fungicide sensitivity between *M. nivale* var. *nivale* and var. *majus* with *M. nivale* var. *nivale* showing reduced sensitivity towards the fungicides used compared to *M. nivale* var. *majus* (Figure 7.4 and Table 7.2). The difference in the sensitivity of isolates of both *M. nivale* sub-species, between fungicides was highly ($P < 0.001$) significant in all cases. Isolates of

both *M. nivale* sub-species were most sensitivity to fludioxonil, followed by bitertanol + fuberidazole, difenoconazole, guazatine, carbendazim + triadimenol with carboxin + thiram being the least active fungicides with respect to rate of active ingredient (Table 7.2). The interaction between sub-species and fungicide showed that *M. nivale* var. *majus* was significantly ($P < 0.05$) more sensitive to Beret Gold than *M. nivale* var. *nivale*.

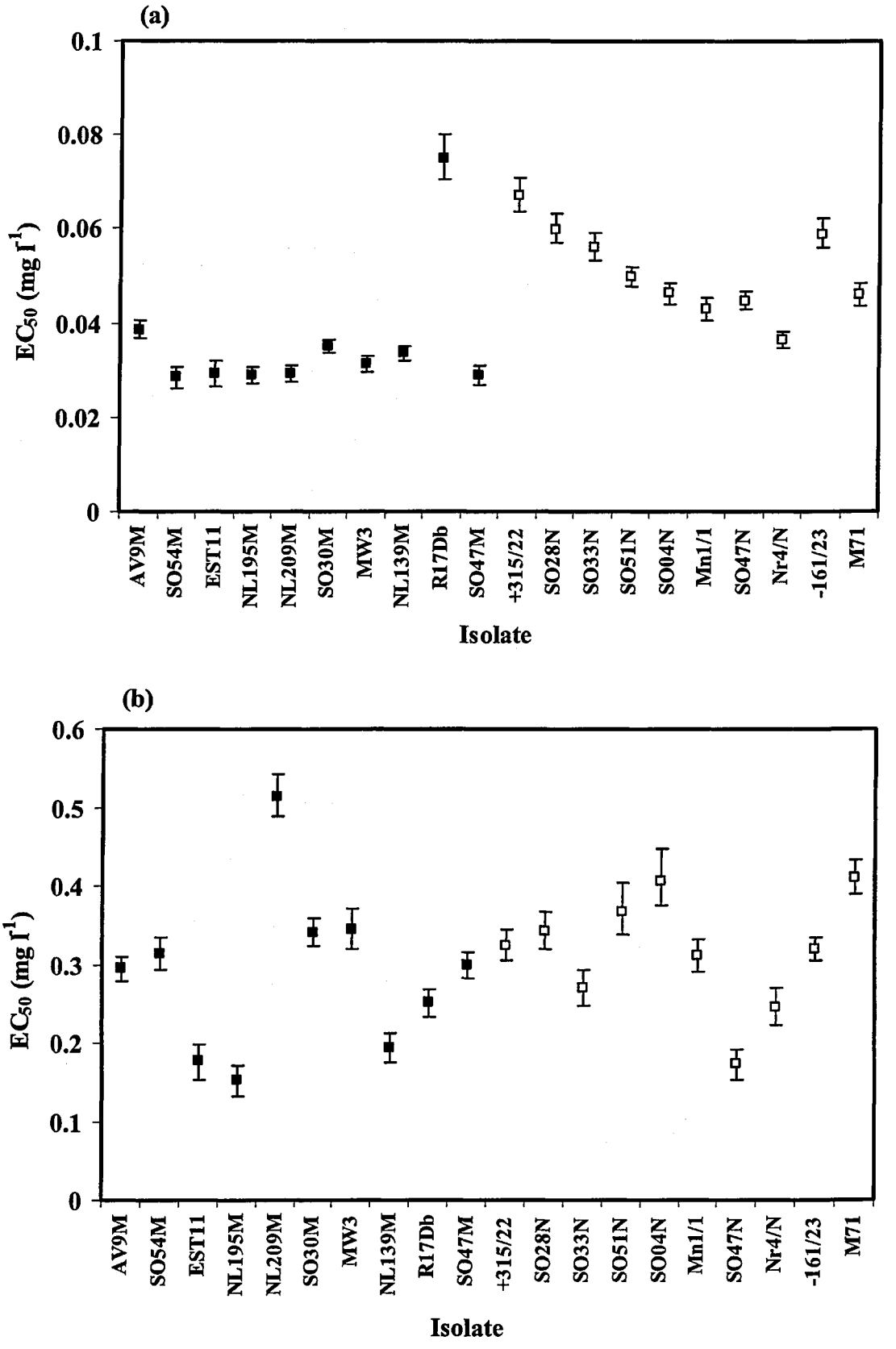


Figure 7.1. Comparison of EC_{50} values for isolates of (■) *M. nivale* var. *majus* and (□) *M. nivale* var. *nivale* determined from radial mycelial growth experiments on PDA plates containing (a) Beret Gold (a.i. fludioxonil) and (b) Sibutol (a.i. bitertanol + fuberidazole). Bars indicate 95% confidence limits.

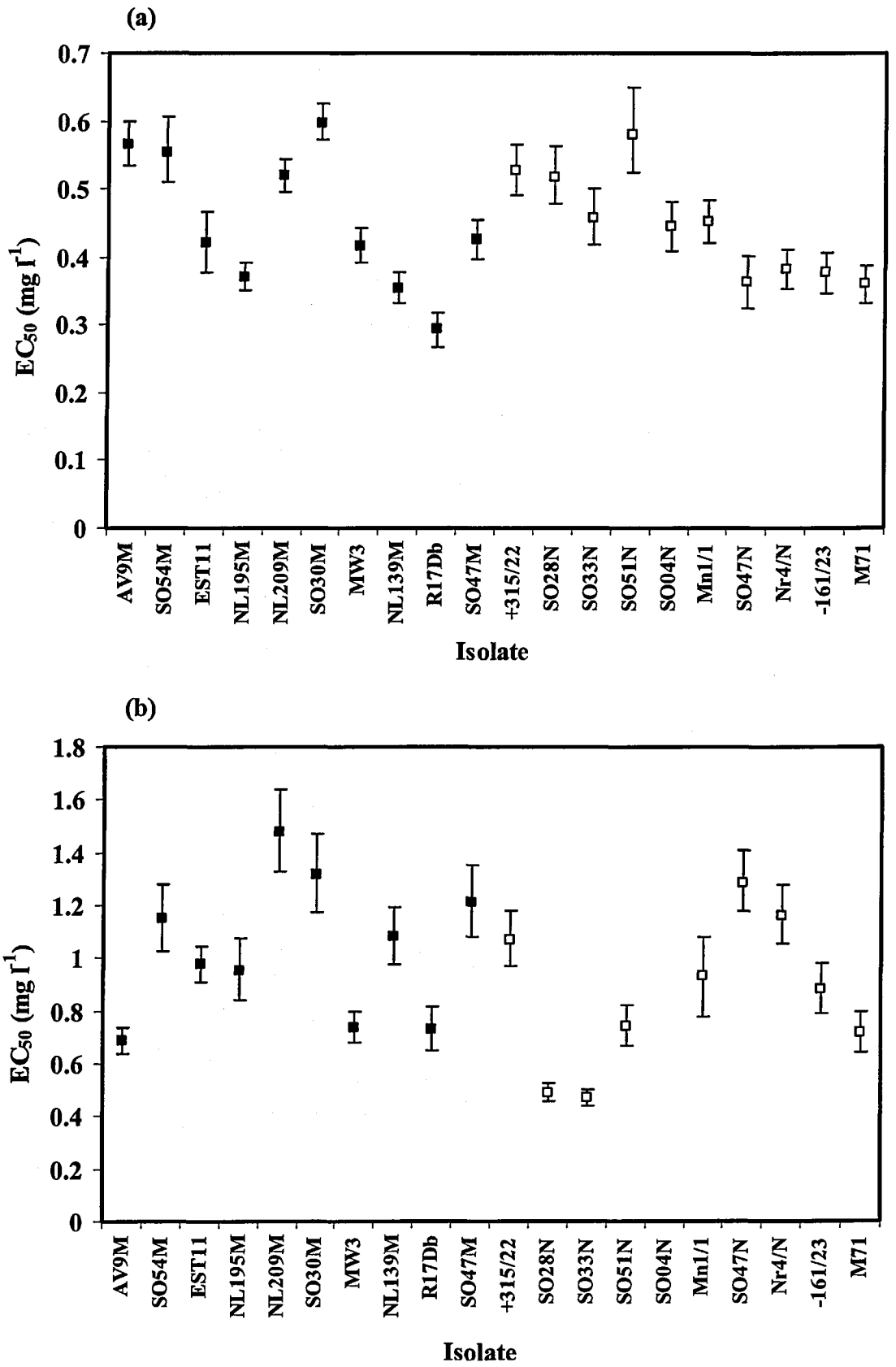


Figure 7.2. Comparison of EC_{50} values for isolates of (■) *M. nivale* var. *majus* and (□) *M. nivale* var. *nivale* determined from radial mycelial growth experiments on PDA plates containing (a) A8179b (a.i. Difenoconazole) and (b) Panoctine (a.i. guazatine + imazalil). Bars indicate 95% confidence limits.

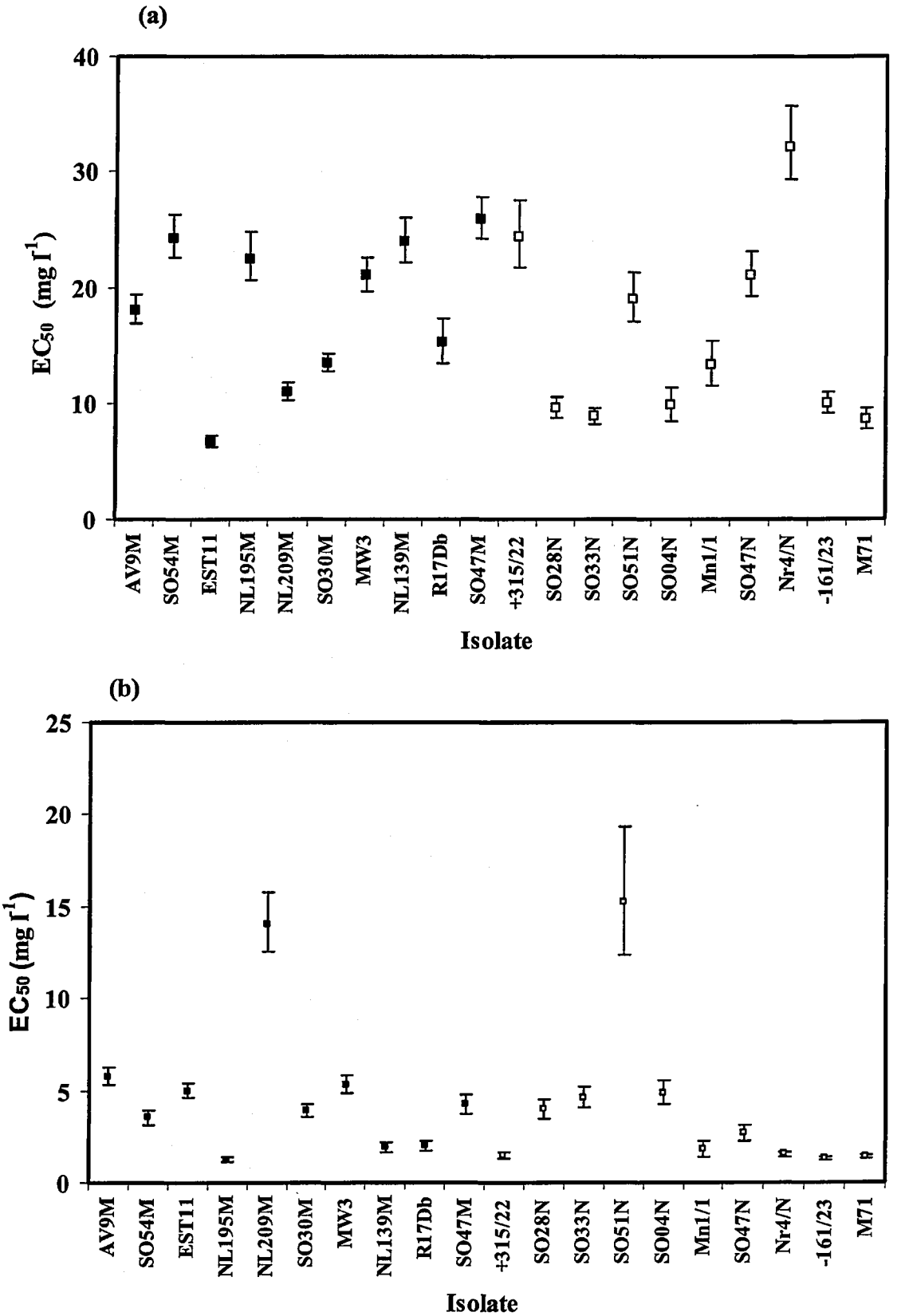


Figure 7.3. Comparison of EC_{50} values for isolates of (■) *M. nivale* var. *majus* and (□) *M. nivale* var. *nivale* determined from radial mycelial growth experiments on PDA plates containing (a) Anchor (a.i. carboxin + thiram) and (b) Baytan (a.i. carbendazim + triadimenol). Bars indicate 95% confidence limits.

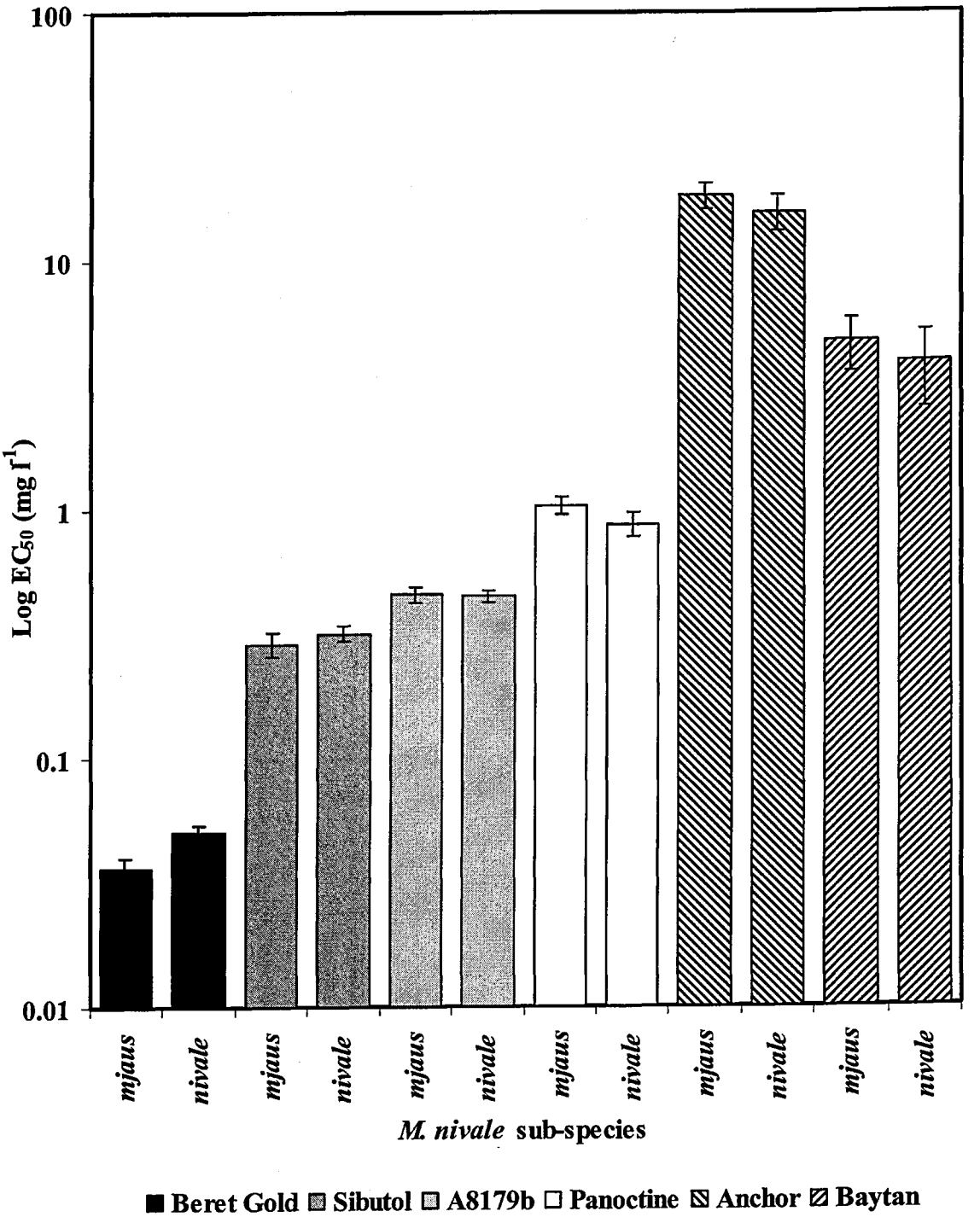


Figure 7.4. Comparison of mean EC_{50} values for *M. nivale* var. *nivale* and var. *majus* determined from radial mycelial growth experiments to six fungicide seed treatments. Bars indicate standard error of mean.

Table 7.2. Comparison of EC_{50} data by two way analysis of variance.

Comparison between sub-species.

Factor	Sub-species	
	M. nivale var. *majus*	*M. nivale* var. *nivale*
Sub-species	1.82	1.64
SEM	0.03	
LSD ($P = 0.05$)	0.08	

Comparison between fungicides

Factor	Beret Gold	Sibutol	Difenoconazole	Panoctine	Anchor	Baytan
Fungicide	5.07	1.88	1.51	1.07	0.26	0.59
SEM	0.05					
LSD ($P = 0.05$)	0.15					

Interaction between sub-species and fungicide

Sub-species/ Fungicide	Beret Gold	Sibutol	Difenoconazole	Panoctine	Anchor	Baytan
var. *majus*	5.66	1.95	1.52	1.01	0.25	0.54
var. *nivale*	4.50	1.82	1.51	1.12	0.27	0.64
SEM	0.07					
LSD ($P = 0.05$)	0.21					

All EC_{50} values transformed using power transformation with constant 0 and m as –0.5. CV = 13.4%, D of F = 117.

EFFECTS OF TEMPERATURE ON THE PERFORMANCE OF THREE FUNGICIDE SEED TREATMENTS TOWARDS SEEDLING BLIGHT OF WHEAT CAUSED BY SEED-BORNE *M. NIVALE* VAR. *NIVALE* AND VAR. *MAJUS*.

Analysis of infected grain

Diagnostic PCR confirmed that no cross-infection between the four seed lots had occurred. From seed lots one and two which were produced from *M. nivale* var. *majus* inoculated plants, only *M. nivale* var. *majus* DNA could be detected. Whereas from seed lots three and four which were produced from *M. nivale* var. *nivale* inoculated plants, only *M. nivale* var. *nivale* DNA could be detected. Quantitative PCR using the JBM primers and the internal standard showed that seed lot one (produced from var. *majus* inoculated plants (5×10^4 spores per ml)) contained 1.13 pg *M. nivale* DNA ng^{-1} total DNA. Seed lot 2 (produced from var. *majus* inoculated plants (2×10^5 spores per ml)) contained 6.67 pg ng^{-1} *M. nivale* DNA. Seed lot 3 (produced from var. *nivale* inoculated plants (5×10^4 spores ml^{-1})) 1.06 pg ng^{-1} *M. nivale*. Seed lot 4 (produced from var. *nivale* inoculated plants (2×10^5 spores per ml)) 4.37 pg ng^{-1} *M. nivale* DNA.

Seedling Emergence

The emergence of seedlings at the four temperatures employed was significantly ($P < 0.05$) lower at 4°C than at the other three temperatures used. No significant ($P < 0.05$) difference was observed in emergence at 8°C or 12°C though emergence was significantly greater at 16°C than at 12°C. No significant ($P > 0.05$) difference was observed in emergence between 8°C and 16°C (Figure 7.5).

The emergence of seedlings produced from fungicide treated seed was significantly ($P < 0.05$) greater than the respective untreated seedlings for all four seed lots; no significant difference was observed in emergence between the seed treatments used (Figure 7.5).

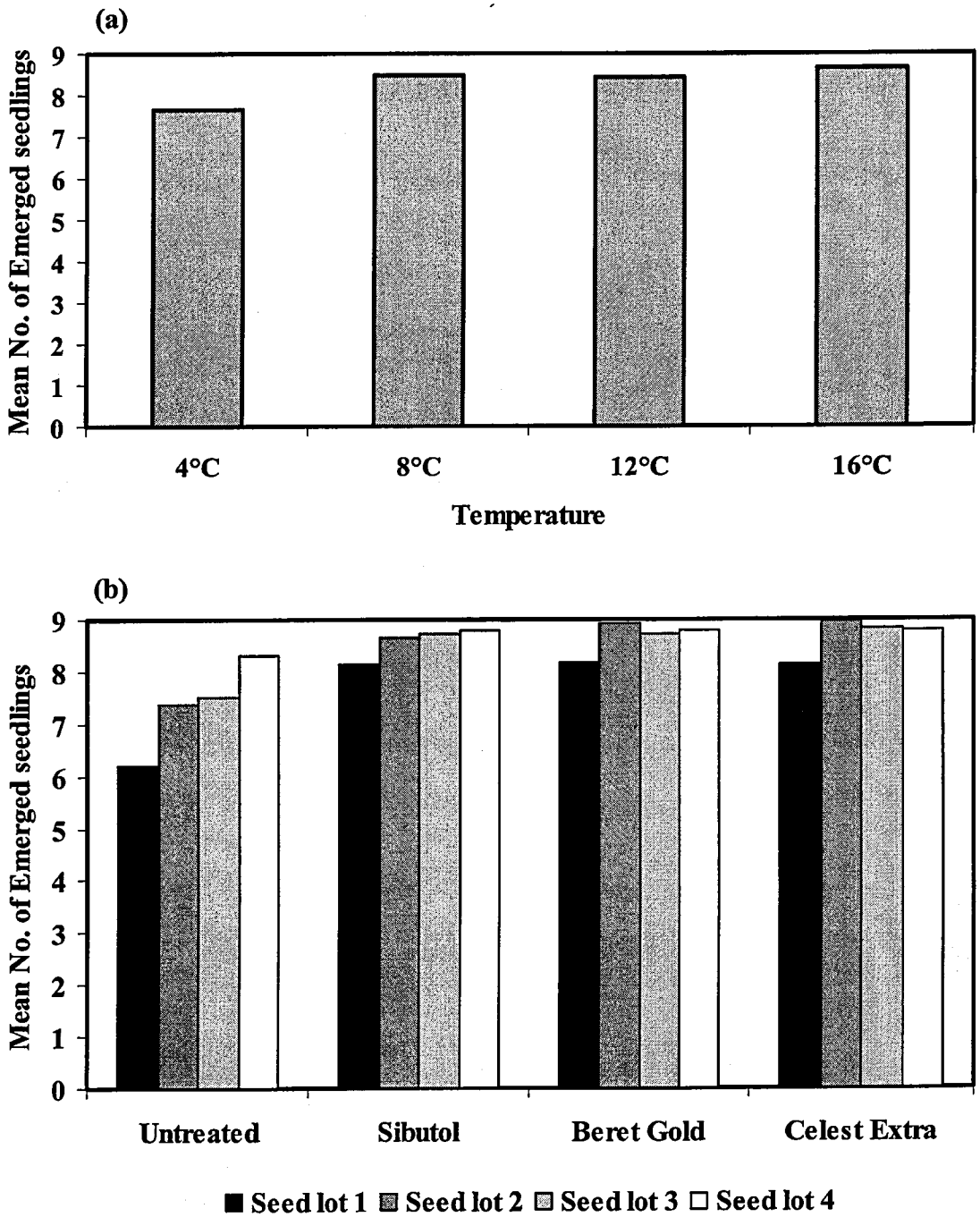


Figure 7.5. The effect of (a) Temperature (SEM = 0.08; LSD (P = 0.05) = 0.22) and (b) Fungicide seed treatment (SEM = 0.16; LSD (P = 0.05) = 0.45) on the emergence of wheat seedlings cv. Cadenza from four seed lots infected with either *M. nivale* var. *nivale* or *M. nivale* var. *majus* (seed lot 1 = var. *majus* infected; 1.13 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 2 = var. *majus* infected; 6.67 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 3 = var. *nivale* infected; 1.06 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 4 = var. *nivale* inected; 4.37 pg *M. nivale* DNA ng^{-1} total DNA. D of F = 511; CV = 11%.

At 4°C, all fungicide seed treatments increased emergence in each of the four seed lots used though no significant ($P > 0.05$) difference between fungicides was observed (Figure 7.6). At 8°C, with the exception of Sibutol treated seed lot 3 (*M. nivale* var. *nivale* infected), all treatments increased emergence significantly ($P < 0.05$) compared to the untreated. No significant ($P > 0.05$) difference was observed in emergence between the untreated and seed treatments used for seed lots 1 (*M. nivale* var. *majus* infected) and 4 (*M. nivale* var. *nivale* infected) at 12°C and seed lots 1,2 (*M. nivale* var. *majus* infected) and 3 at 16°C (Figure 7.6). For seed lots 2 and 3 at 12°C and seed lot 4 at 16°C the three seed treatments used increased emergence significantly ($P < 0.05$).

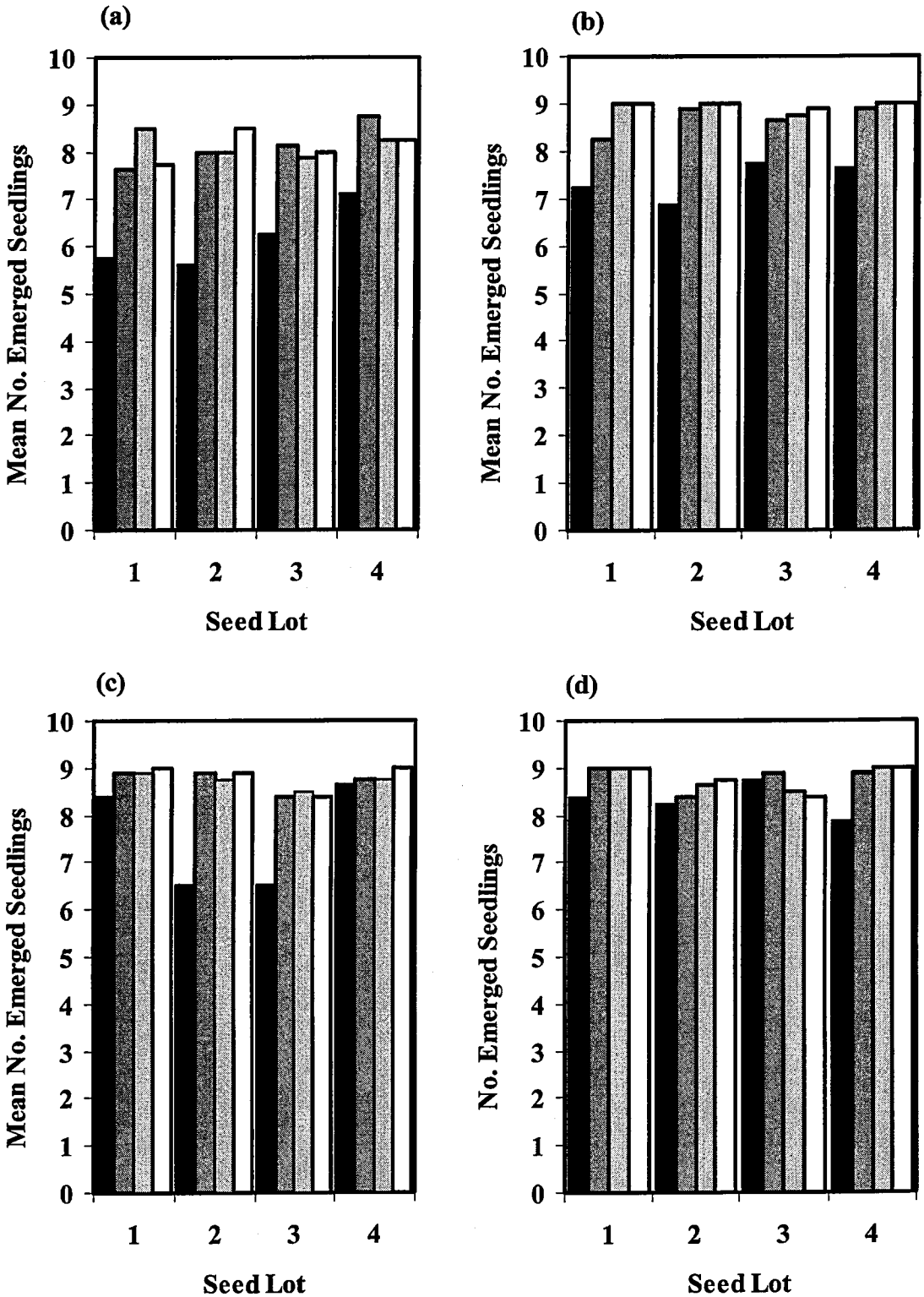


Figure 7.6. Effect of temperature (a) 4°C, (b) 8°C, (c) 12°C, (d) 16°C and fungicide seed treatment (■ = untreated, ▩ = Sibutol, ▨ = Beret Gold, □ = Celest Extra) (SEM = 0.32; LSD (P = 0.05) = 0.89) on the emergence of wheat seedlings cv. Cadenza from four seed lots infected with either *M. nivale* var. *nivale* or *M. nivale* var. *majus* (seed lot 1 = var. *majus* infected; 1.13 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 2 = var. *majus* infected; 6.67 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 3 = var. *nivale* infected; 1.06 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 4 = var. *nivale* inected; 4.37 pg *M. nivale* DNA ng^{-1} total DNA.D of F = 511; CV = 11%.

Stem-base disease severity

The severity of stem-base symptoms was significantly ($P < 0.05$) greater at 8°C and 12°C than at 4°C; the least severe symptoms were observed on seedlings produced at 16°C (Figure 7.7a). No significant ($P > 0.05$) difference was observed in the severity of stem-base symptoms produced from seedlings treated with either Beret Gold or Celest Extra from any of the four seed lots used (Figure 7.7b). Symptom severity was significantly ($P < 0.05$) greater in seedlings produced from Sibutol treated seed than Beret Gold or Celest Extra in each of the four seed lots. No significant ($P > 0.05$) difference was observed in the severity of stem-base symptoms produced from the four seed lots with the exception that Sibutol treated seed from lot 2 produced seedlings with significantly ($P < 0.05$) more severe symptoms than those from the other three seed lots used. Untreated seedlings from seed lot 2 produced seedlings with significantly ($P < 0.05$) more severe stem-base symptoms than those produced from untreated seedlings from the other three seed lots. The severity of stem-base symptoms produced from untreated seed lots one and three did not differ significantly ($P < 0.05$), untreated seedlings from seed lot two were significantly ($P < 0.05$) different to those from the untreated for seed lots one and two but not three. The severity of stem-base symptoms was reduced significantly ($P < 0.05$) by all three seed treatments for all four seed lots at each of the four temperatures employed (Figure 7.8). No significant ($P > 0.05$) difference was observed between seed treatments used in any of the four seed lots at 4°C. At 8°C, Beret Gold and Celest Extra significantly ($P < 0.05$) reduced the severity of stem-base symptoms more than Sibutol for seed lots one and two.

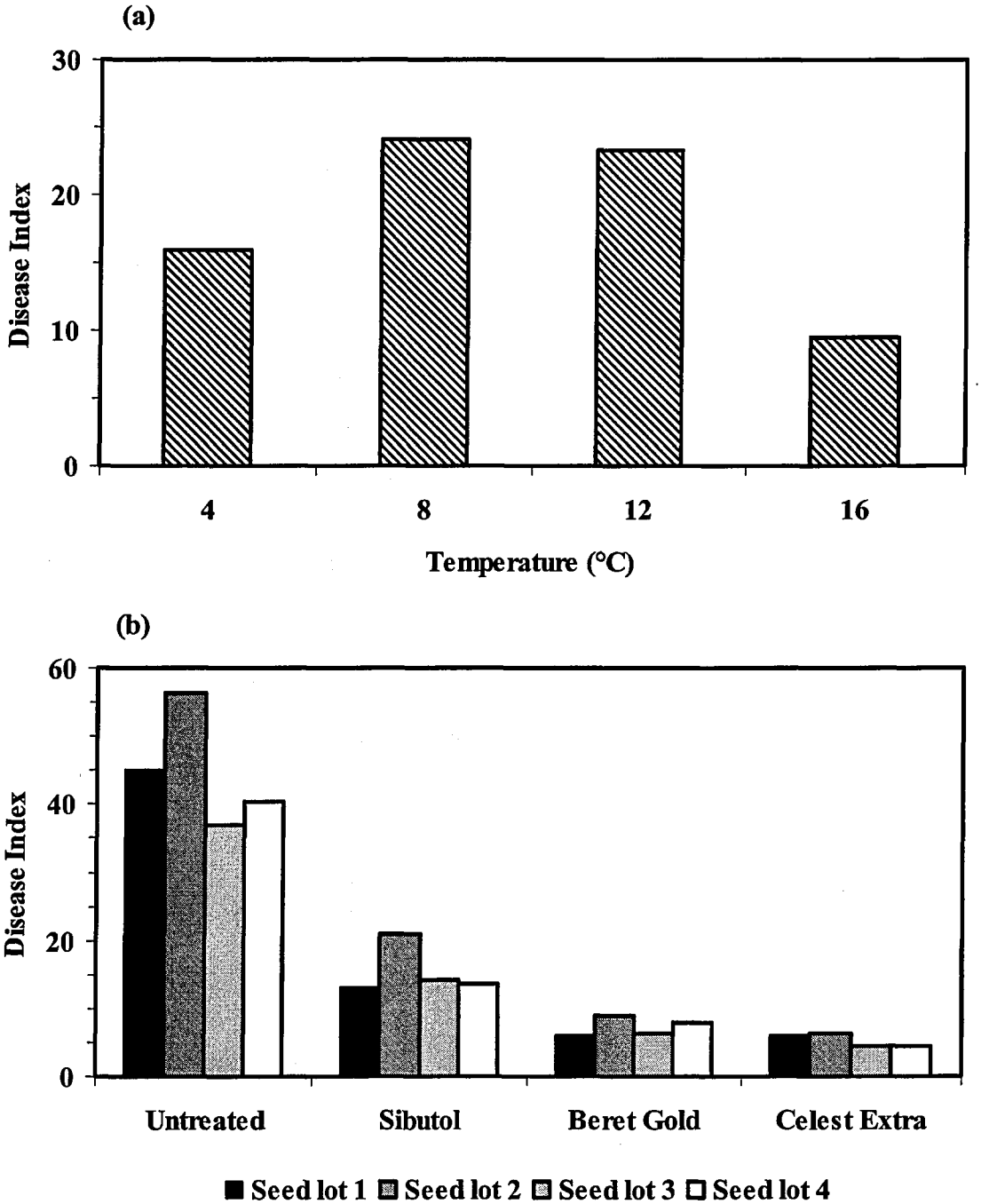


Figure 7.7. The effect of (a) Temperature (SEM = 1.78; LSD (P = 0.05) = 2.47) and (b) Fungicide seed treatment (SEM = 0.89; LSD (P = 0.05) = 4.93) on disease severity of emerged wheat seedlings cv. Cadenza from four seed lots infected with either *M. nivale* var. *nivale* or *M. nivale* var. *majus* (seed lot 1 = var. *majus* infected; 1.13 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 2 = var. *majus* infected; 6.67 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 3 = var. *nivale* infected; 1.06 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 4 = var. *nivale* inected; 4.37 pg *M. nivale* DNA ng^{-1} total DNA. D of F = 511; CV = 55%.

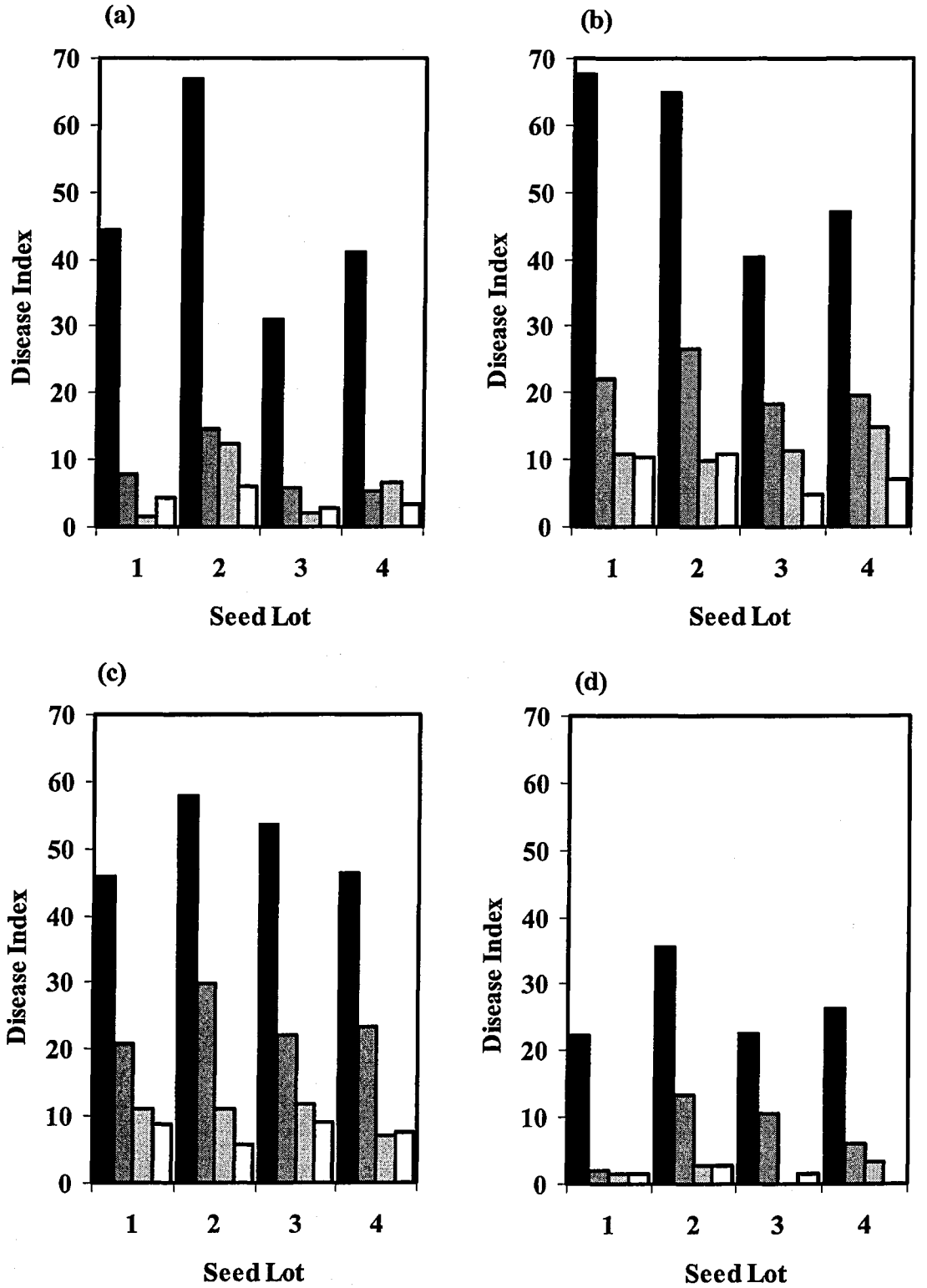


Figure 7.8. Effect of temperature (a) 4°C, (b) 8°C, (c) 12°C, (d) 16°C and fungicide seed treatment (■ = untreated, ▩ = Sibutol, ▨ = Beret Gold, □ = Celest Extra) and temperature (SEM = 3.55; LSD (P = 0.05) = 9.86) on disease severity on emerged seedlings cv. Cadenza from four seed lots infected with either *M. nivale* var. *nivale* or *M. nivale* var. *majus* (seed lot 1 = var. *majus* infected; 1.13 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 2 = var. *majus* infected; 6.67 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 3 = var. *nivale* infected; 1.06 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 4 = var. *nivale* inected; 4.37 pg *M. nivale* DNA ng^{-1} total DNA. D of F = 511; CV = 55%.

For seed lots 3 and 4, the severity of symptoms was reduced significantly ($P < 0.05$) more by Celest Extra than Sibutol though no significant ($P < 0.05$) difference was observed between Sibutol and Beret Gold or between Beret Gold and Celest Extra. At 12°C, Beret Gold and Celest Extra reduced the severity of stem-base symptoms significantly ($P < 0.05$) more than Sibutol and also for seed lots 2 and 3 at 16°C. For seed lots 1 and 4 at 16°C, no significant ($P < 0.05$) difference was observed between seed treatments.

Symptom incidence

The incidence of seedlings showing symptoms of stem-base infections was greatest at 8°C though was not significantly different to that at 12°C. The incidence of infection was significantly ($P < 0.05$) lower at 16°C than at 4°C which was significantly lower than at 8°C or 12°C.

All seed treatments used reduced the incidence of stem-base symptoms significantly ($P < 0.05$) compared to untreated seed for all four seed lots (Table 7.3). Beret Gold and Celest Extra provided the greatest reduction in the incidence of stem-base symptoms though they were not significantly ($P > 0.05$) different to one another for any of the four seed lots. Beret Gold and Celest Extra reduced the incidence of stem-base symptoms significantly ($P < 0.05$) compared to Sibutol in all four seed lots.

All seed treatments reduced the incidence of stem-base symptoms significantly ($P < 0.05$) compared to the control for all four seed lots at the four temperatures used with the exception of seed lots 2 and 3 at 12°C (Table 7.3). No significant ($P > 0.05$) difference was observed between Beret Gold and Sibutol at any temperature for any of the seed lots used with the exception that Celest Extra reduced the incidence of symptoms significantly compared to Beret Gold for seed lots 3 and 4 at 8°C. Beret Gold and Celest Extra reduced the incidence of stem-base symptoms significantly ($P < 0.05$) compared to Sibutol for seed

Table 7.3. Effect of (a) temperature, (b) fungicide seed treatment (c) temperature and fungicides seed treatment on the incidence of stem-base seedling blight symptoms on seedlings from four seed lots cv. Cadenza infected with either *M. nivale* var. *nivale* or *M. nivale* var. *majus* (seed lot 1 = var. *majus* infected; 1.13 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 2 = var. *majus* infected; 6.67 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 3 = var. *nivale* infected; 1.06 pg *M. nivale* DNA ng^{-1} total DNA, seed lot 4 = var. *nivale* inected; 4.37 pg *M. nivale* DNA ng^{-1} total DNA. Transformed values in parenthesis.

(a)

No. infected seedlings (mean)			
4°C	8°C	12°C	16°
1.53 (-3.92)	2.87 (-3.44)	2.94 (-3.44)	1.11 (-4.08)

SEM = 0.04; LSD (P = 0.05) = 0.11

(b)

Seed Lot	No. Infected seedlings (mean)			
	Untreated	Sibutol	Beret Gold	Celest Extra
1	4.84 (-2.89)	1.66 (-3.79)	0.72 (-4.18)	0.59 (-4.20)
2	5.75 (-2.67)	2.97 (-3.39)	1.06 (-4.05)	0.69 (-4.18)
3	3.91 (-3.10)	1.78 (-3.70)	0.78 (-4.18)	0.50 (-4.30)
4	5.34 (-2.76)	1.81 (-3.75)	0.88 (-4.09)	0.50 (-4.30)

SEM = 0.08; LSD (P = 0.05) = 0.22

(c)

Treatment	Seed Lot	No. infected seedlings (mean)			
		4°C	8°C	12°C	16°C
Untreated	1	3.50 (-3.12)	7.00 (-2.45)	6.13 (-2.58)	2.75 (-3.41)
Sibutol	2	0.75 (-4.10)	2.75 (-3.26)	3.00 (-3.29)	0.13 (-4.51)
Beret Gold	3	0.13 (-4.51)	1.25 (-3.86)	1.38 (-3.84)	0.13 (-4.51)
Celest Extra	4	0.38 (-4.33)	1.00 (-3.93)	0.88 (-4.05)	0.13 (-4.51)
Untreated	1	5.13 (-2.80)	6.88 (-2.46)	6.25 (-2.59)	4.75 (-2.82)
Sibutol	2	1.63 (-3.77)	4.38 (-2.99)	4.38 (-2.99)	1.50 (-3.79)
Beret Gold	3	1.25 (-3.93)	1.25 (-3.91)	1.50 (-3.92)	0.25 (-4.42)
Celest Extra	4	0.63 (-4.23)	1.13 (-3.88)	0.75 (-4.19)	0.25 (-4.42)
Untreated	1	3.13 (-3.29)	5.00 (-2.90)	5.00 (-2.78)	2.50 (-3.41)
Sibutol	2	0.50 (-4.24)	2.38 (-3.42)	3.25 (-3.15)	1.00 (-3.98)
Beret Gold	3	0.25 (-4.46)	1.38 (-3.82)	1.50 (-3.84)	0.00 (-4.60)
Celest Extra	4	0.25 (-4.42)	0.50 (-4.28)	1.13 (-4.00)	0.13 (-4.51)
Untreated	1	5.38 (-2.71)	6.00 (-2.64)	6.63 (-2.52)	3.38 (-3.17)
Sibutol	2	0.63 (-4.24)	2.63 (-3.41)	3.38 (-3.15)	0.63 (-4.19)
Beret Gold	3	0.63 (-4.19)	1.75 (-3.61)	0.88 (-4.13)	0.25 (-4.42)
Celest Extra	4	0.38 (-4.37)	0.63 (-4.16)	1.00 (-4.07)	0.00 (-4.60)

SEM = 0.16; LSD (P = 0.05) = 0.44; D of F = 511; CV = 12%.

lot 1 at 8°C and 12°C, at all temperatures for seed lot 2, at 12°C and 16°C for seed lot 3 and at 12°C for seed lot 4. For all other seed lot and temperature combinations, no significant ($P > 0.05$) difference was observed between the three seed treatments used. Diagnostic PCR showed that no cross-contamination between seedlings produced from either var. *nivale* or var. *majus* infected seed had occurred.

FIELD TRIALS

Analysis of seed produced

Germination

The germination of seed produced in 1998 and 1999 improved significantly ($P < 0.05$) through the use of either Sibutol or Beret Gold at 4°C and 20°C across the four seed lots. In 1999, Beret Gold improved germination significantly ($P < 0.05$) more than Sibutol across the four seed lots.

At 4°C, all 1998 seed lots showed significantly ($P < 0.05$) different germination with seed lot 1 giving the highest followed by seed lot 4, seed lot 3 and 2 (Figure 7.9). For 1998 seed, at 20°C, germination was significantly ($P < 0.05$) greatest in seed lot 1 and lowest for seed lot 2 (Figure 7.9).

In 1999, at 4°C, seed lot 1 showed significantly ($P < 0.05$) greater germination than the other three seed lots, seed lot 2 showed the lowest germination at 4°C (Figure 7.10). At 20°C, all seed lots showed significantly ($P < 0.05$) different germination, seed lot 1 gave the greatest germination followed by seed lot 3, 4 and 2 (Figure 7.10).

For 1998 seed, both seed treatments significantly ($P < 0.05$) improved germination compared to the untreated for all seed lots with the exception of seed lot 1 at 4°C for which germination was not significantly ($P > 0.05$) improved by either treatment. For 1999 seed, the germination of the four seed lots was significantly ($P < 0.05$) improved at both 4°C and 20°C using either seed treatment.

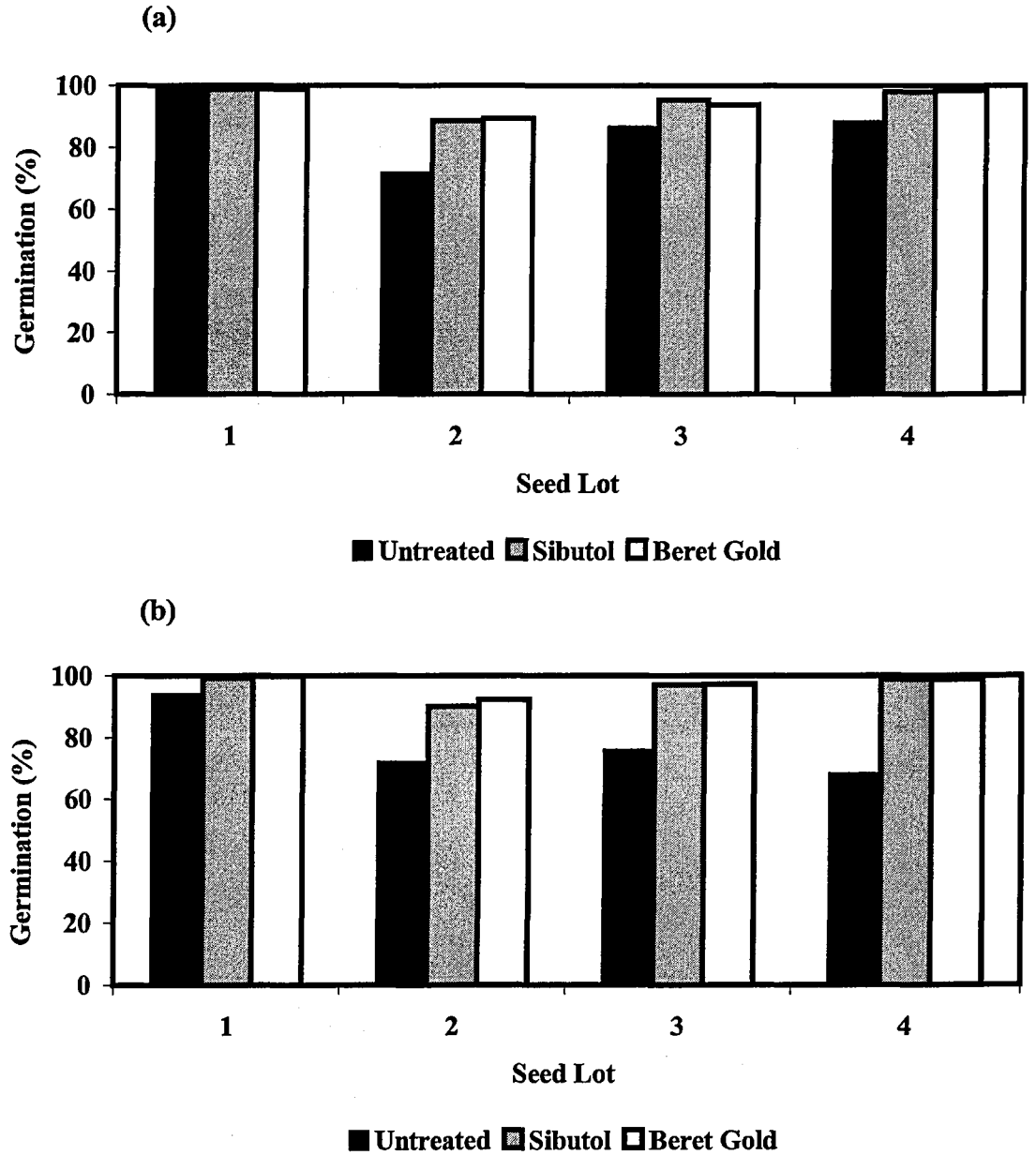


Figure 7.9. The effect of fungicide seed treatment and Seed Lot on germination at (a) 4°C (SEM = 0.682; LSD (P = 0.05) = 1.956; D of F = 47; CV = 1.5%) and (b) 20°C (SEM = 1.007; LSD (*P* = 0.05) = 2.888; D of F = 47; CV = 2.2%) of wheat seed cv. Hussar used in 1998 seedling blight trial.

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1998**; seed Lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *F. graminearum*; seed lot 4 = *M. nivale* var. *majus*.

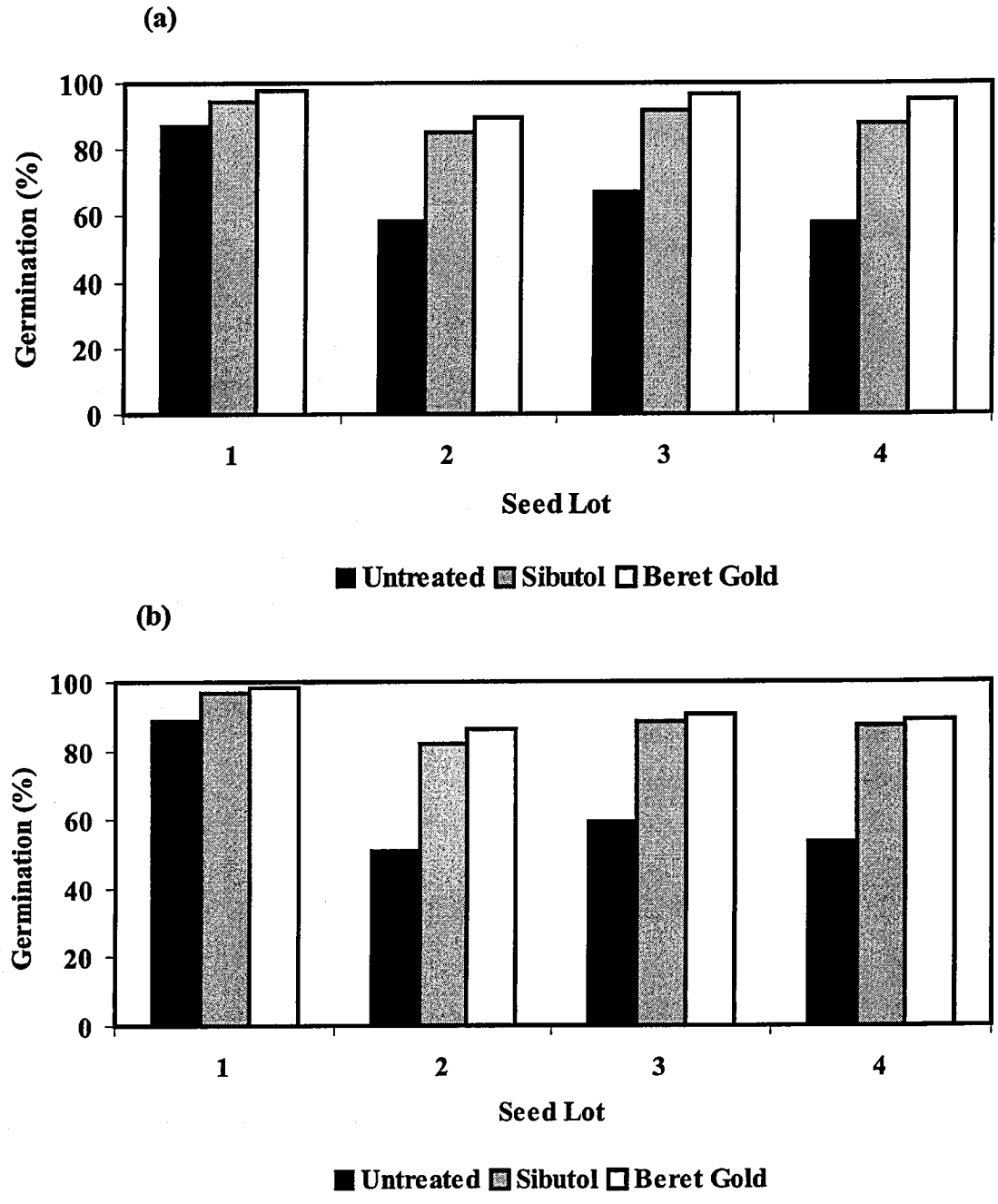


Figure 7.10. The effect of fungicide seed treatment and Seed Lot on germination at (a) 4°C (SEM = 1.524; LSD (P = 0.05) = 4.371; D of F = 47; CV = 3.8%) and (b) 20°C (SEM = 1.943; LSD (P = 0.05) = 5.573; D of F = 47; CV = 4.6%) of wheat seed cv. Equinox used in 1999 seedling blight trial.

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

Incidence of M. nivale *var.* nivale, M. nivale *var.* majus *and* Fusarium *spp.*

In both years, seed produced from field plots inoculated with *Fusarium* spp. or *M. nivale* resulted in seed with percentage infections of that pathogen which were greater than that in the commercial seed (lot 1). In both years, seed lot 2 had the highest incidence of *Fusarium* spp. infection. Seed lot 4 had the highest incidence of *M. nivale* in 1998 and seed lot 2 the lowest (Figure 7.11). For the 1999 seed, seed lots 3 and 4 had the highest incidence of *M. nivale* with a similar incidence of the two *M. nivale* sub-species followed by seed lots 2 and 1 (Figure 7.12).

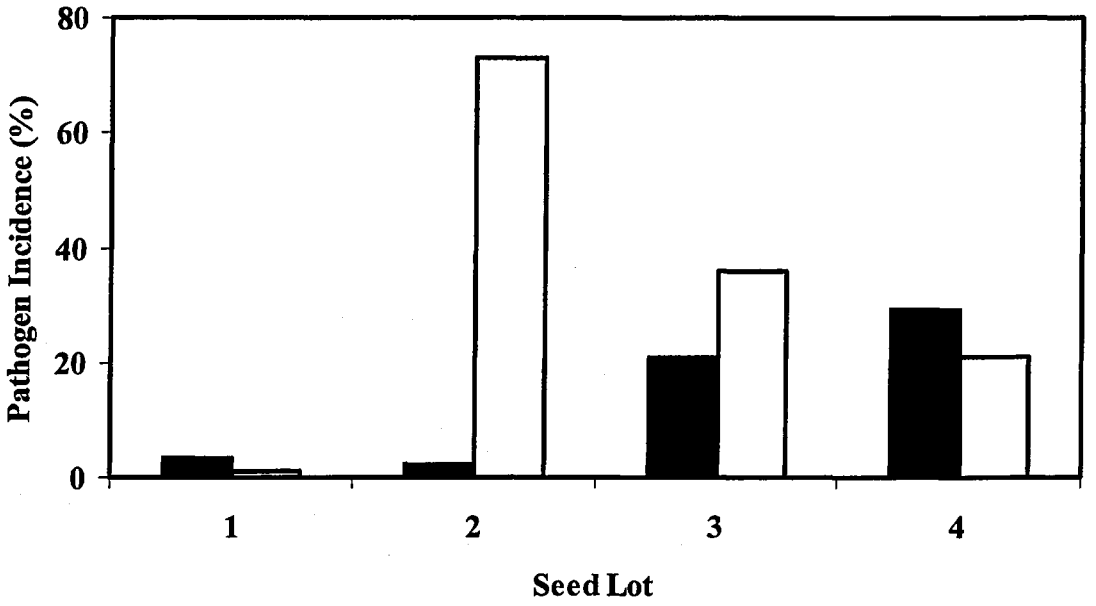


Figure 7.11. Percentage of wheat seeds cv. Hussar infected with (■) *M. nivale* and (□) *Fusarium* spp. (determined by agar plate counts) in four seed lots used for 1998 field trial.

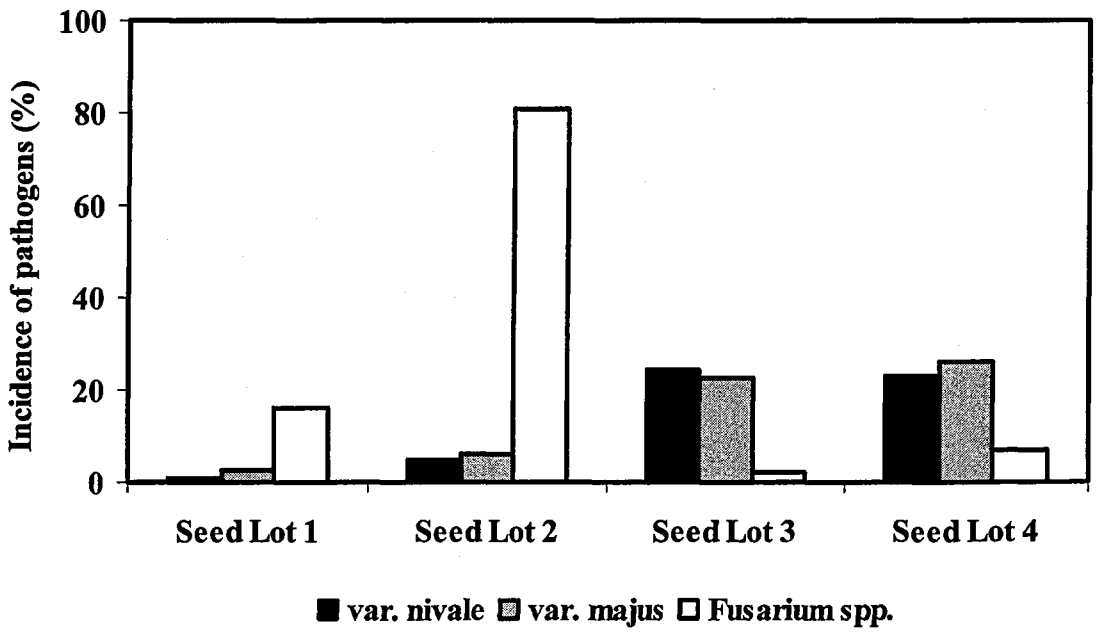


Figure 7.12. Percentage of wheat seeds cv. Equinox infected with *M. nivale* var. *nivale*, var. *majus* and *Fusarium* spp. (determined by agar plate counts; PCR analysis used to determine *M. nivale* sub-species) in four seed lots used for 1999 field trial.

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis: **1998**; seed Lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *F. graminearum*; seed lot 4 = *M. nivale* var. *majus*. **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

Quantification of M. nivale *var.* nivale *and var.* majus *in 1998 and 1999 seed*

Both *M. nivale* sub-species were detected using the PCR assays in all four seed lots used in both 1998 and 1999 (Figure 7.13 and 7.14). In 1998 significantly ($P < 0.05$) more *M. nivale* var. *nivale* than var. *majus* was detected across the seed lots (Table 7.4). In 1999, no significant ($P > 0.05$) difference was observed between the amount of *M. nivale* var. *nivale* and var. *majus* across the seed lots (Table 7.5). In both years the total amount of *M. nivale* detected was significantly ($P < 0.05$) different between all four seed lots used. Seed lot 1 contained the least *M. nivale* followed by seed lots 2, 3 and 4 which contained the most *M. nivale.*

For seed lots 1 and 4 in 1998 and seed lot 4 in 1999, significantly ($P < 0.05$) more *M. nivale* var. *nivale* was detected than var. *majus*. For all other seed lots in both years, there was no significant ($P > 0.05$) difference between the amount of *M. nivale* var. *nivale* and var. *majus* detected.

Quantification of Fusarium *spp. in 1998 and 1999 seed*

Significantly ($P < 0.05$) more *Fusarium* spp. DNA was detected in 1999 than in 1998 across the four seed lots used in each year (Figure 7.15). The amount of *Fusarium* spp. detected in the four seed lots across the years was significantly ($P < 0.05$) different in all cases, seed lot 2 contained the most, followed by seed lots 3, 4 and 1 (Table 7.6).

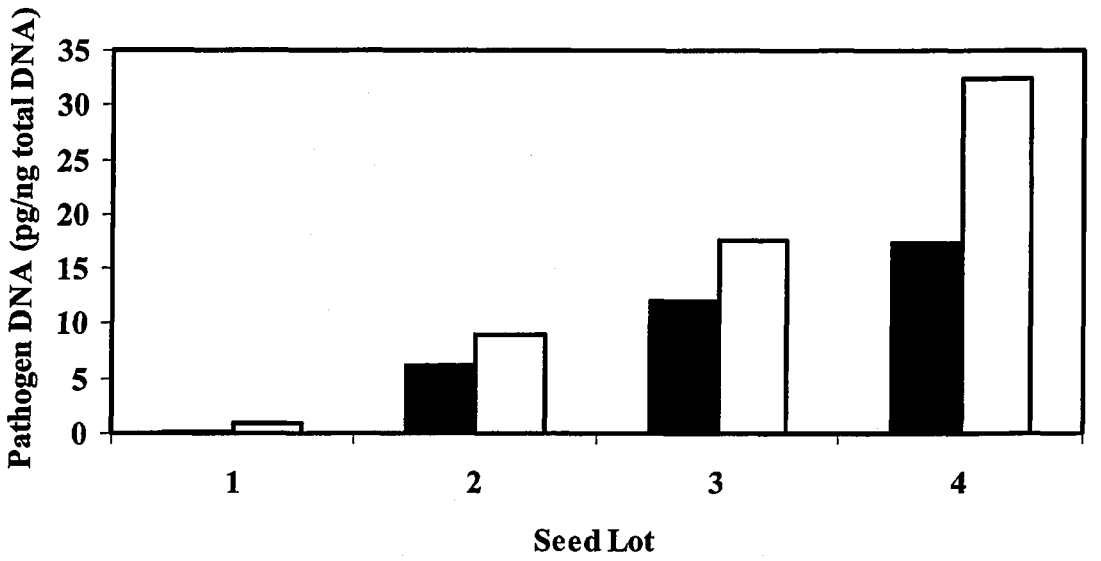


Figure 7.13. PCR quantification of (□) *M. nivale* var. *nivale* and (■) *M. nivale* var. *majus* for four seed lots used in 1998 field trial. Statistical analysis results given in Table 7.4 (below).

Table 7.4. Statistical analysis of *M. nivale* var. *nivale* and var. *majus* quantitative PCR data for 1998 field trial seed.

Sub-species / Log pathogen DNA (pg ng^{-1} total DNA)	
M. nivale var. *majus*	*M. nivale* var. *nivale*
0.55	0.91

F. Probability = < 0.001; SEM = 0.04; LSD (*P*= 0.05) = 0.10

Seed Lot / Log pathogen DNA (pg ng^{-1} total DNA)			
1	2	3	4
-0.49	0.87	1.16	1.36

F. Probability = < 0.001; SEM = 0.05; LSD (P = 0.05) = 0.15

Sub-species	Seed Lot / Log pathogen DNA (pg ng^{-1} total DNA)			
	1	2	3	4
var. *majus*	-0.92	0.79	1.08	1.22
var. *nivale*	-0.07	0.95	1.24	1.50

F. Probability = < 0.001; SEM = 0.07; LSD (P = 0.05) = 0.21

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1998**; seed Lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *F. graminearum*; seed lot 4 = *M. nivale* var. *majus*.

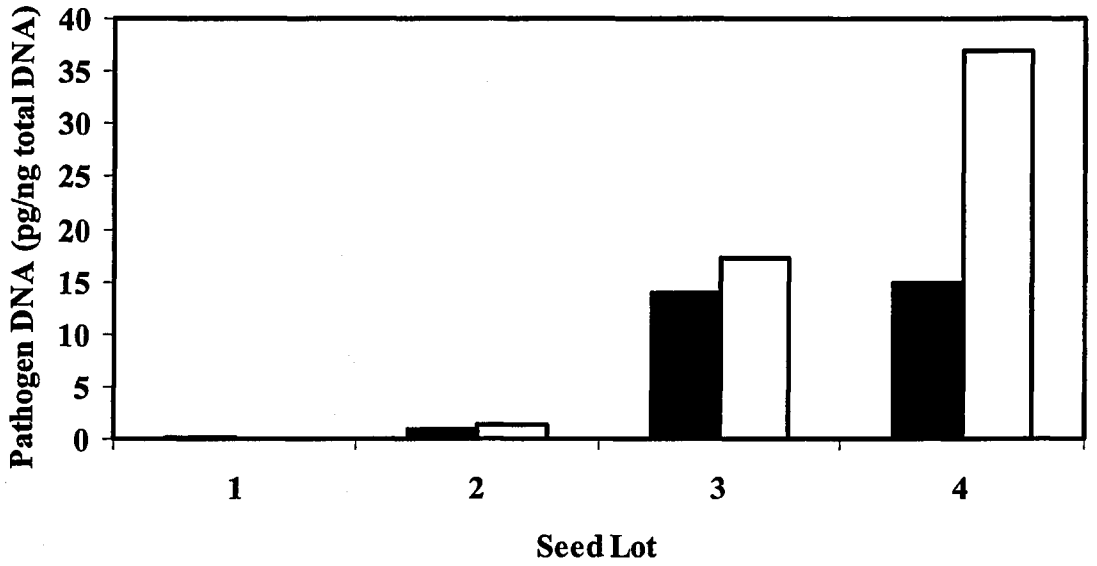


Figure 7.14. PCR quantification of (□) *M. nivale* var. *nivale* and (■) *M. nivale* var. *majus* for four seed lots used in 1999 field trial. Statistical analysis results given in Table 7.5 (below).

Table 7.5. Statistical analysis of *M. nivale* var. *nivale* and var. *majus* quantitative PCR data for 1999 field trial seed.

Sub-species / Log pathogen DNA (pg ng^{-1} total DNA)	
M. nivale var. *majus*	*M. nivale* var. *nivale*
0.30	0.42

F. Probability = 0.05; SEM = 0.04; LSD (P = 0.05) = 0.12

Seed Lot / Log pathogen DNA (pg ng^{-1} total DNA)			
1	2	3	4
-1.15	0.03	1.19	1.37

F. Probability = < 0.001; SEM = 0.06; LSD (P = 0.05) = 0.17

Sub-species	Seed Lot / Log pathogen DNA (pg ng^{-1} total DNA)			
	1	2	3	4
var. *majus*	-1.05	-0.06	1.14	1.17
var. *nivale*	-1.25	0.12	1.24	1.57

F. Probability = 0.01; SEM = 0.08; LSD (P = 0.05) = 0.24; D of F = 31; CV = 45%

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

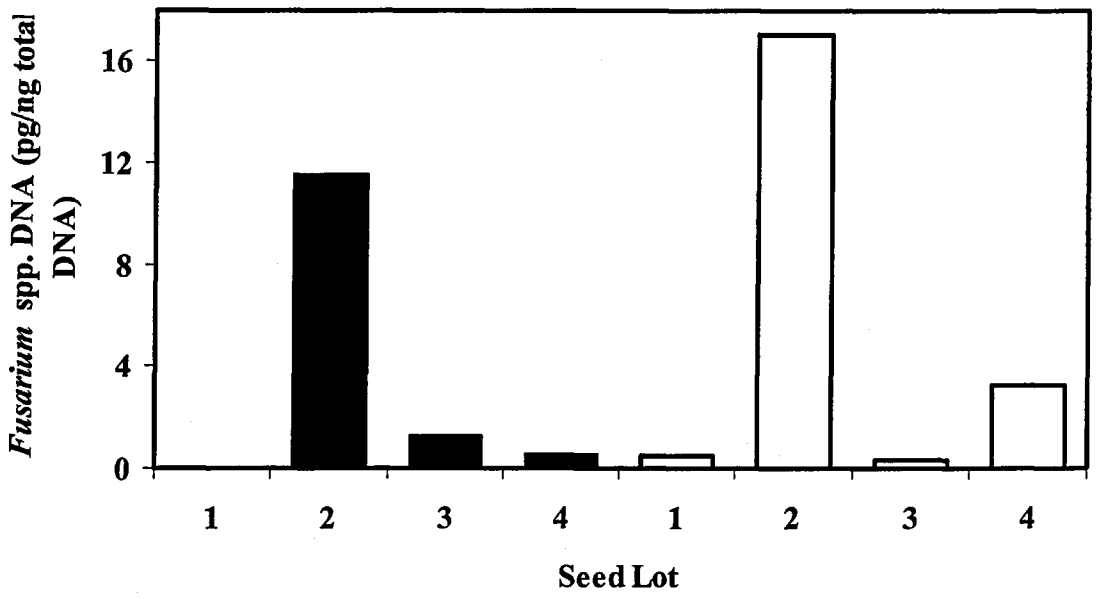


Figure 7.15. PCR quantification of *Fusarium* spp. in field trial seed in (■) 1998 and (□) 1999. Statistical analysis results given below.

Table 7.6. Statistical analysis of *Fusarium* spp. quantitative PCR data for 1998 and 1999 field trial seed.

Year / Power (m = -0.5) *Fusarium* spp. DNA	
1998	1999
2.75	1.01

F. Probability = < 0.001; SEM = 0.04; LSD (P = 0.05) = 0.12

Seed Lot / Power (m = -0.5) *Fusarium* spp. DNA			
1	2	3	4
4.977	0.27	1.33	0.9

F. Probability = < 0.001; SEM = 0.06; LSD (P = 0.05) = 0.18

Year	Seed Lot / Power (m = -0.5) *Fusarium* spp. DNA			
	1	2	3	4
1998	8.48	0.30	0.90	1.34
1999	1.48	0.25	1.76	0.56

F. Probability = < 0.001; SEM = 0.09; LSD (P = 0.05) = 0.25
D of F = 31; CV = 9.0%.

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis: **1998**; seed Lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *F. graminearum*; seed lot 4 = *M. nivale* var. *majus*. **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

Analysis of field trial plots and seedlings

Stand counts

Stand counts for the 1998 and 1999 field trials showed no significant ($P < 0.05$) difference in the number of emerged seedlings from seed lot 1 with either Sibutol or Beret Gold seed treatments compared to the untreated at either GS12 or GS25 across the seed lots (Tables 7.7 and 7.8). In 1998 at GS12 Sibutol and Beret Gold both significantly ($P < 0.05$) increased emergence compared to the untreated for seed lots 2 and 4 (Table 7.7). For seed lot 3, Beret Gold treated seed was significantly greater than both the untreated and Sibutol treated seed. For seed lot 4, Beret Gold significantly ($P < 0.05$) improved emergence compared to Sibutol treated seed. At GS25, no significant ($P > 0.05$) difference was observed in seedling emergence from treated and untreated seed for seed lots 1, 2 and 4 however, for seed lot 3, Beret Gold significantly ($P < 0.05$) improved emergence compared to both the untreated and Sibutol treated seed. In the 1999 trial, at both GS12 and GS25, both Sibutol and Beret Gold significantly increased the number of emerged seedlings compared to the untreated for seed lots 2, 3 and 4 (Table 7.8). Beret Gold increased emergence significantly ($P < 0.05$) more than Sibutol for seed lot 4 at GS25.

Stem-base disease severity

At GS12, stem-base disease severity was reduced significantly ($P < 0.05$) by both Sibutol and Beret Gold compared to the respective untreated for seed lots 2, 3 and 4 in both 1998 and 1999 (Figure 7.16 and 7.17). Beret Gold reduced stem-base disease severity significantly ($P < 0.05$) more than Sibutol at GS12 for seed lots 3 and 4 in 1999. No significant ($P > 0.05$) reduction in stem-base disease severity was observed in either 1998 or 1999 for seed lot 1.

Table 7.7. Mean stand counts taken at growth stages 12 and 25 for seedlings produced from four seed lots cv. Hussar treated with either Sibutol or Beret Gold (untreated seed used as a control) and drilled at Edgmond in 1998.

Treatment	Seed Lot / mean plants per m row Assessment 1 (GS12)				Seed lot / mean plants per m row Assessment 2 (GS25)			
	1	2	3	4	1	2	3	4
Untreated	34.2	18.9	20.0	25.7	21.4	16.3	16.4	18.6
Sibutol	37.6	32.3	24.2	36.1	20.6	19.4	17.6	20.9
Beret Gold	36.0	29.2	32.0	42.0	21.3	18.6	20.5	21.7

Assessment 1; F. Probability (Treatment*Seed) Lot = 0.002; SEM = 1.8; LSD ($P = 0.05$) = 5.2; D of F = 47; CV = 11.7%.
Assessment 2; F. Probability (Treatment*Seed) Lot = <0.001; SEM = 1.1; LSD ($P = 0.05$) = 3.1; D of F = 47; CV = 11.1%.

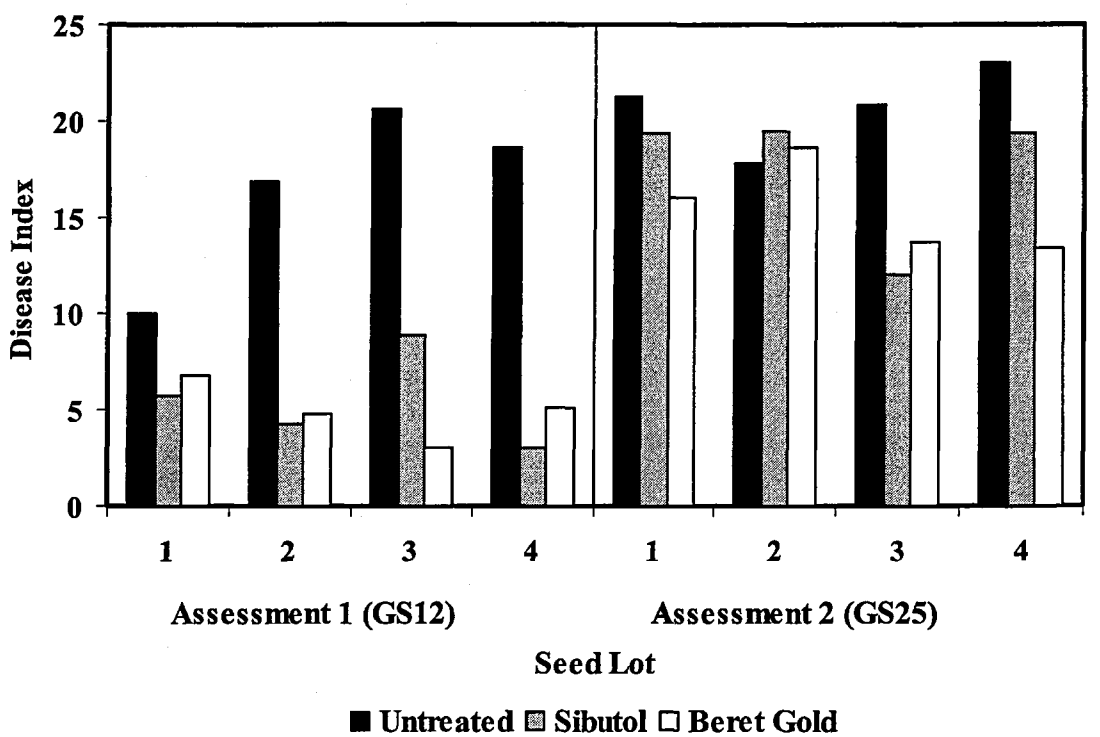


Figure 7.16. Seedling blight disease severity at growth stage 12 and 25 in seedlings produced from four seed lots with two fungicide seed treatments drilled at Edgmond in 1998. Assessment 1; SEM = 2.5; LSD (P = 0.05) = 7.3; D of F = 47; CV = 56.3%. Assessment 2; SEM = 2.1; LSD (P = 0.05) = 6.1; D of F = 47; CV = 23.7%.

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1998**; seed Lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *F. graminearum*; seed lot 4 = *M. nivale* var. *majus*.

Table 7.8. Mean stand counts taken at growth stages 12 and 25 for seedlings produced from four seed lots cv. Equinox treated with either Sibutol or Beret Gold (untreated seed used as a control) and drilled at Edgmond in 1999.

Treatment	Seed Lot / plants per m row Assessment 1 (GS12)				Seed lot / plants per m row Assessment 2 (GS25)			
	1	2	3	4	1	2	3	4
Untreated	45.9	19.4	18.1	19.0	46.4	16.7	17.4	16.9
Sibutol	45.1	29.8	35.5	35.1	45.2	28.8	33.7	33.2
Beret Gold	43.2	28.4	36.8	37.8	44.3	27.7	34.3	36.6

Assessment 1; F. Probability (Treatment*Seed) Lot = <0.001; SEM = 1.1; LSD (P = 0.05) = 3.1; D of F = 47; CV = 6.6%.
Assessment 2; F. Probability (Treatment*Seed) Lot = <0.001; SEM = 1.0; LSD (P = 0.05) = 3.0; D of F = 47; CV = 6.5%.

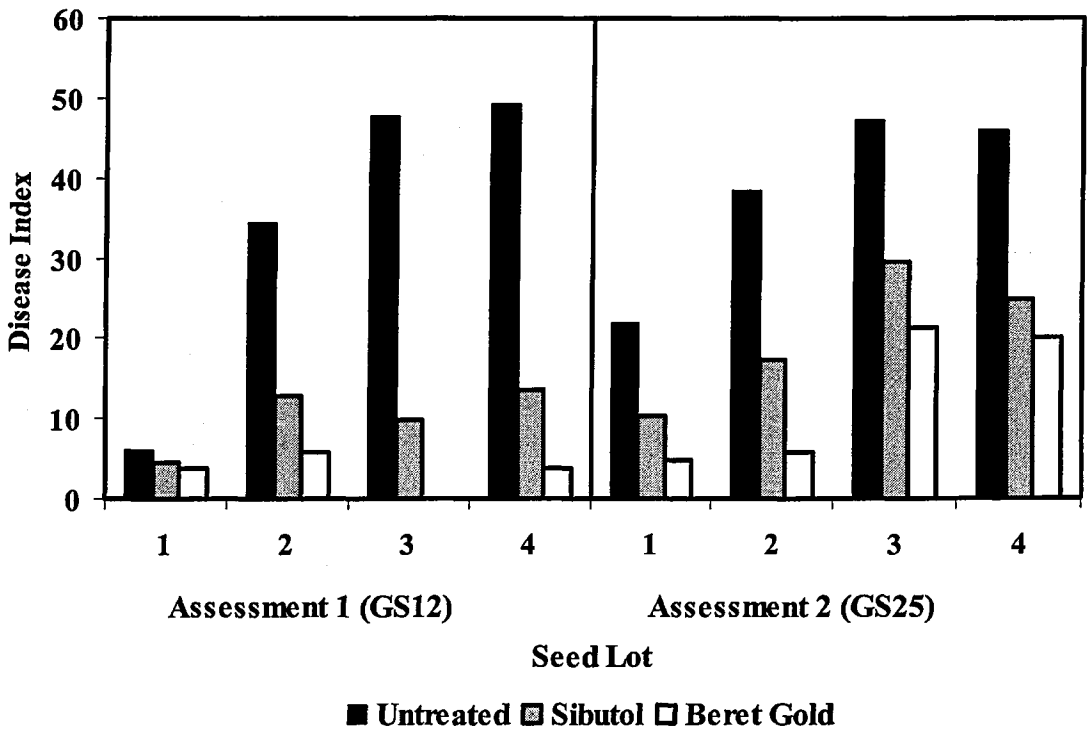


Figure 7.17. Seedling blight disease severity at growth stage 12 and 25 in seedlings produced from four seed lots with two fungicide seed treatments drilled at Edgmond in 1999. Assessment 1; SEM = 2.8; LSD (P = 0.05) = 8.1; D of F = 47; CV = 35.6%. Assessment 2; SEM = 2.9; LSD (P = 0.05) = 8.5; D of F = 47; CV = 24.6%.

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

At GS25, in 1998, stem-base disease severity was reduced significantly ($P < 0.05$) by Sibutol and Beret Gold compared to the untreated for seed lots 1 and 3. For seed lot 2, no significant ($P > 0.05$) difference was observed between treatments and for seed lot 4, stem-base disease severity was reduced significantly ($P < 0.05$) compared to the untreated by Beret Gold but not Sibutol. No significant ($P < 0.05$) difference was observed between Sibutol and Beret Gold. At GS25, stem-base disease severity was reduced significantly ($P < 0.05$) by either seed treatment compared to the untreated for all four seed lots. Beret Gold reduced stem-base disease severity significantly more than Sibutol for seed lots 2 and 3.

For the 1999 trial, at GS59, stem-base disease severity was significantly ($P < 0.05$) lower on plants produced from Beret Gold treated seed compared to the those produced from either untreated or Sibutol treated seed across the seed lots (Figure 7.18). No significant ($P < 0.05$) difference was observed in disease severity between seed lots or between treatments for seed lots 1 and 3. For seed lots 2 and 4, plants produced from Beret Gold treated seed showed significantly ($P < 0.05$) less severe stem-base symptoms compared to those produced from Sibutol treated seed. For seed lot 4 the severity of symptoms on plants produced from Beret Gold treated seed did not differ significantly ($P < 0.05$) from those observed on plants produced from untreated seed.

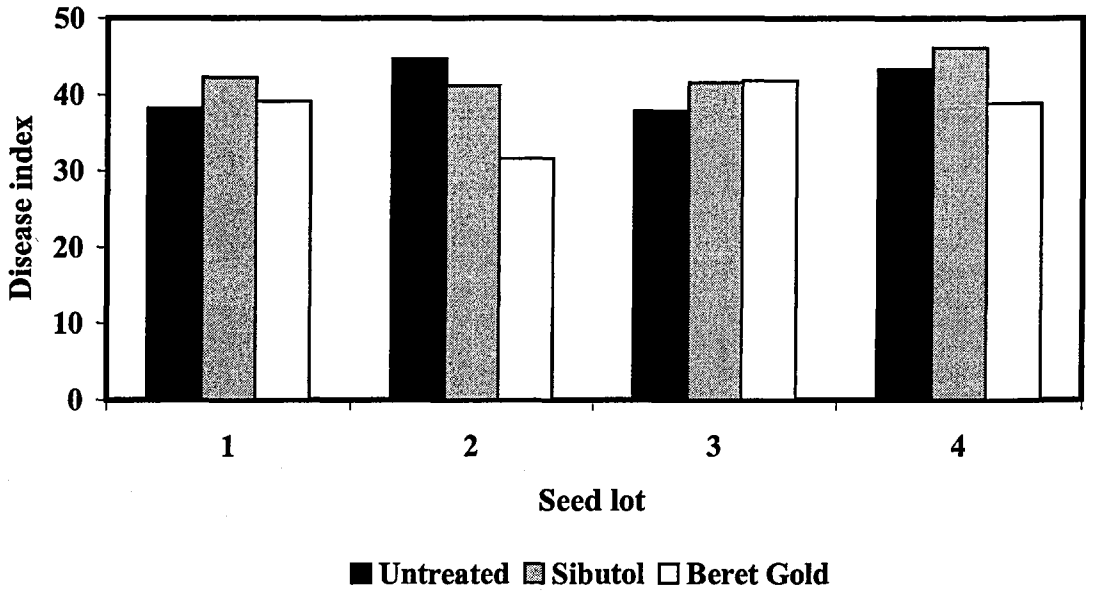


Figure 7.18. The effect of fungicide seed treatment and seed lot. on the severity of stem-base disease in winter wheat plant cv. Equinox at GS59 in 1999. (SEM = 2.665; LSD (P = 0.05) = 5.422) D of F = 47; CV = 9.3%; CV, CV (block) = 8.7%.

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

Quantitative PCR results for 1998 and 1999 field trials

Accumulated analysis of variance using the generalised linear model showed there to be no significant ($P < 0.05$) difference between replicate PCR's for the quantification of *M. nivale* or *Fusarium* spp (Appenix 13).

Quantification of M. nivale *and* Fusarium *spp. in seedlings from 1998 trial*

At GS12, *M. nivale* was detected in samples from all plots, the amount of *M. nivale* quantified in seedlings was reduced significantly ($P < 0.05$) using either seed treatment compared to the untreated across the seed lots (Figure 7.19). Beret Gold reduced the amount of *M. nivale* DNA significantly ($P < 0.05$) more than Sibutol across the seed lots. The amount of *M. nivale* detected was lowest in seedlings produced from seed lot 1 and greatest in seedlings produced from seed lots 3 and 4. Beret Gold and Sibutol both

significantly ($P < 0.05$) reduced the amount of *M. nivale* in seedlings from seed lots 2, 3 and 4 but not in seedlings produced from seed lot 1. Beret Gold reduced the amount of *M. nivale* significantly ($P < 0.05$) compared to Sibutol in seedlings produced from seed lot 3. At GS25, the amount of *M. nivale* in seedlings was reduced significantly by either seed treatment across the seed lots, no significant ($P > 0.05$) difference was observed between the seed treatments (Figure 7.20). The amount of *M. nivale* detected in seedlings from seed lots 1 and 2 was significantly lower than that detected in seedlings from seed lots 3 and 4. Both seed treatments significantly ($P < 0.05$) reduced the amount of *M. nivale* in seedlings from seed lots 1, 3 and 4, no significant ($P > 0.05$) difference was observed between seed treatments in these seed lots.

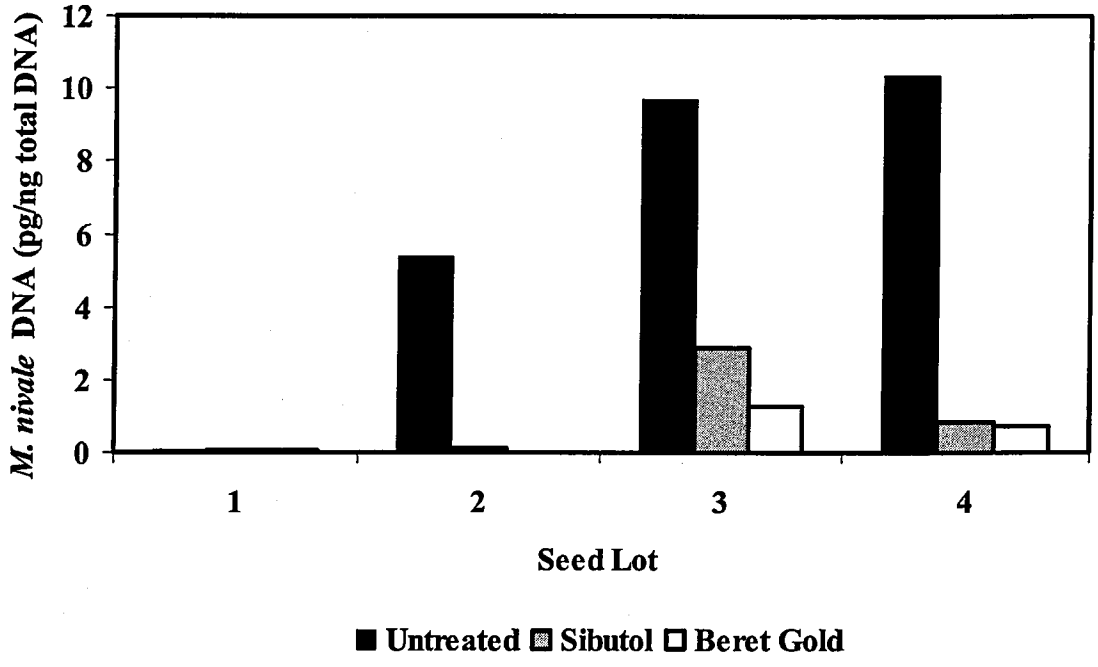


Figure 7.19. Effect of the use of two seed treatments on the quantity of *M. nivale* DNA in wheat seedlings cv. Hussar at growth stage 12 produced from four separate seed lots in 1998 (see Table 7.9 for statistical analysis).

Table 7.9. Statistical analysis of *M. nivale* DNA data following Log (DNA+1) transformation.

Treatment	Log (*M. nivale* DNA + 1)/ Seed Lot				
	1	2	3	4	All
Untreated	0.03	0.76	1.00	1.03	0.71
Sibutol	0.03	0.04	0.51	0.22	0.20
Beret Gold	0.02	0.01	0.29	0.20	0.13
All	0.03	0.27	0.60	0.48	

N.B. Transformation; Log (*M. nivale* DNA +1) was used in order to normalise data prior to statistical analysis.

Seed Lot: SEM = 0.03; LSD ($P = 0.05$) = 0.07; $P = <0.001$
Treatment: SEM = 0.03; LSD ($P = 0.05$) = 0.06; $P = <0.001$
Treatment*Seed Lot: SEM = 0.06; LSD ($P = 0.05$) = 0.12; $P = <0.001$
D of F = 134: CV = 42%; CV (block) = 18.0%

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1998**; seed Lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *F. graminearum*; seed lot 4 = *M. nivale* var. *majus*.

Figure 7.20. Effect of the use of two seed treatments on the quantity of *M. nivale* DNA in wheat seedlings cv. Hussar at growth stage 25 produced from four separate seed lots in 1998 (see Table 7.10 for statistical analysis).

Table 7.10. Statistical analysis of *M. nivale* DNA data following Log (DNA+1) transformation.

Treatment	Log (*M. nivale* DNA + 1)/ Seed Lot				
	1	2	3	4	All
Untreated	0.70	0.64	0.86	1.01	0.80
Sibutol	0.36	0.57	0.61	0.54	0.52
Beret Gold	0.42	0.41	0.55	0.48	0.47
All	0.49	0.54	0.67	0.68	

N.B. Transformation; Log (*M. nivale* DNA +1) was used in order to normalise data prior to statistical analysis.

Seed Lot; SEM = 0.04; LSD (143 D of F) = 0.07; $P = <0.001$
Treatment; SEM = 0.03; LSD (143 D of F) = 0.06; $P = <0.001$
Treatment*Seed Lot; SEM = 0.07; LSD (143 D of F) = 0.13; $P = <0.001$
CV = 26.6%;CV (block) = 3.2%

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1998**; seed Lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *F. graminearum*; seed lot 4 = *M. nivale* var. *majus*.

The amount of *M. nivale* in Sibutol treated seedlings from seed lot 2 did not differ significantly ($P < 0.05$) from the untreated and was significantly ($P < 0.05$) more than that detected in seedlings produced from Beret Gold treated seed.

At GS12, *Fusarium* spp. were only detected in seedlings sampled from untreated seed from seed lot 2 (34.0 pg ng^{-1}); at GS25. *Fusarium* spp. could not be detected in any seedling samples from any plots.

Incidence of M. nivale *var.* nivale, M. nivale *var.* majus *and* Fusarium *spp. on seedlings at GS12 from the 1999 trial*

The incidence of *M. nivale* var. *nivale*, var. *majus* and *Fusarium* spp. from seedlings at GS12 showed that significantly ($P < 0.05$) more *Fusarium* spp. isolates were present on seedlings across the four seed lots and three treatments than *M. nivale* var. *nivale*. No significant difference was observed between the incidence of the two *M. nivale* sub-species or between *M. nivale* var. *majus* and *Fusarium* spp. (Figure 7.21). No significant ($P > 0.05$) difference in the incidence of the three pathogens was observed for seedlings produced from seed lot 1. Significantly ($P < 0.05$) fewer isolations of *M. nivale* var. *nivale* and var. *majus* were made from seedlings produced from Sibutol or Beret Gold treated seed for seed lots 2, 3 and 4 compared to the untreated, though no significant ($P > 0.05$) difference between treatments was observed. No significant ($P > 0.05$) difference was observed on the incidence of *Fusarium* spp. isolates from seedlings produced from treated or untreated seed for seed lots 1 and 4 though both treatments significantly reduced the incidence of *Fusarium* spp. for seed lot 2. For seed lot 3, the incidence of *Fusarium* spp. was reduced significantly ($P < 0.05$) by Sibutol but not Beret Gold compared to the untreated.

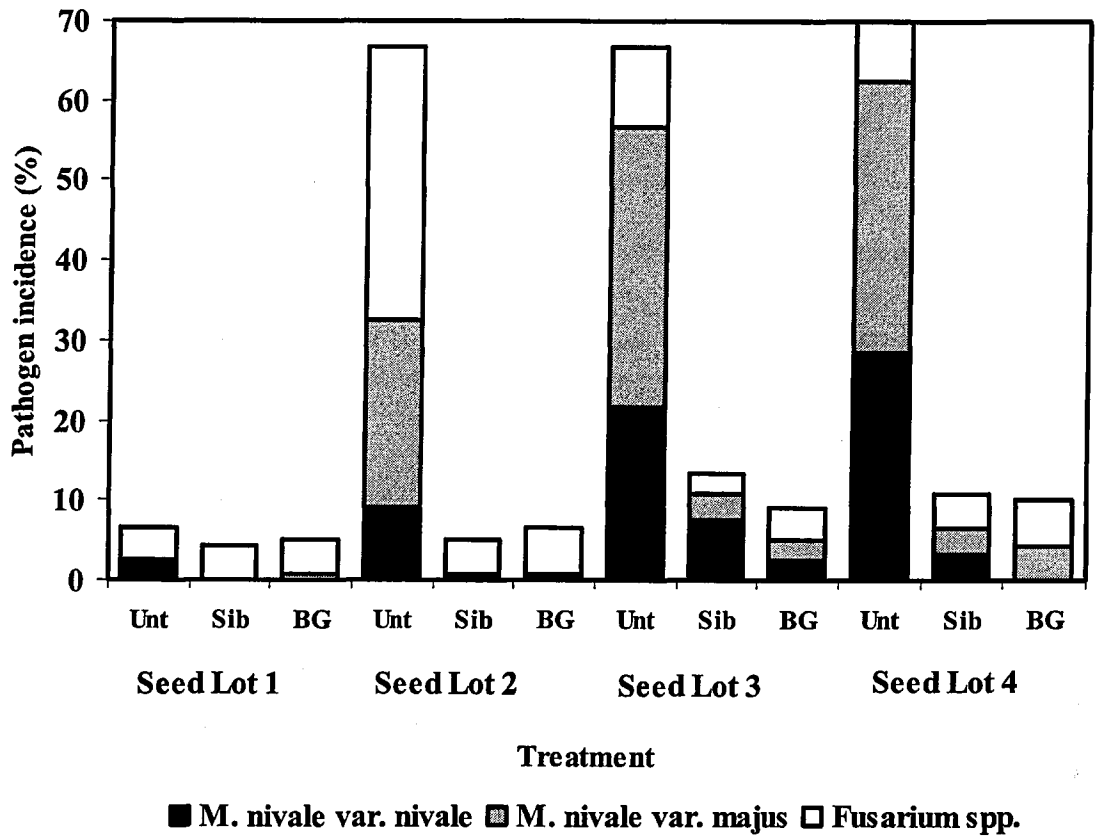


Figure 7.21. Incidence of *M. nivale* var. *nivale*, var. *majus* and *Fusarium* spp. from seedlings (GS12) produced from treated and untreated winter wheat seed lots cv. Equinox in 1999. Unt = untreated; Sib = Sibutol; BG = Beret Gold.

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

Quantification of M. nivale *and* Fusarium *spp. in 1999 field trial*

Microdochium nivale was detected at GS12 in seedlings produced from all plots. The amount of *M. nivale* was reduced significantly ($P < 0.05$) by both Sibutol or Beret Gold across the four seed lots and Beret Gold reduced the amount of *M. nivale* significantly ($P < 0.05$) more than Sibutol (Figure 7.22). The amount of *M. nivale* in seedlings produced from seed lot 1 was significantly ($P < 0.05$) less than those from seed lot 2 which were significantly ($P < 0.05$) less than those produced from seed lots 3 and 4.

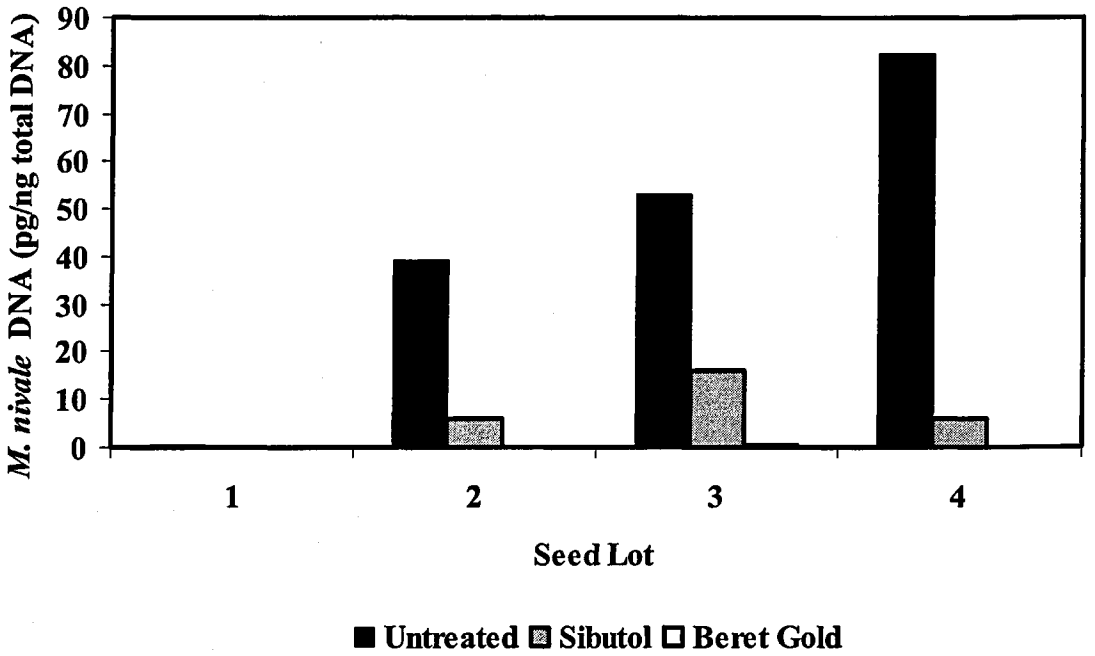


Figure 7.22. Comparison of *M. nivale* DNA quantified in seedlings at GS12 produced from treated and untreated seed from four seed lots in 1999 (see Table 7.11 for statistical analysis).

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

Table 7.11. Statistical analysis of *M. nivale* DNA data following Log transformation.

Treatment	Log *M. nivale* DNA / Seed lot				
	1	2	3	4	All
Untreated	0.10	1.50	1.71	1.92	1.31
Sibutol	0.00	0.72	1.07	0.78	0.64
Beret Gold	0.01	0.01	0.10	0.02	0.03
All	0.04	0.74	0.96	0.90	

N.B. *M. nivale* DNA data was Log transformed in order to normalise data prior to statistical analysis.

Seed Lot; SEM = 0.04; LSD = 0.09; $P = <0.001$
Treatment; SEM = 0.04; LSD = 0.08; $P = <0.001$
Treatment*Seed Lot; SEM = 0.06; LSD = 0.12; $P = <0.001$
D of F = 143; CV = 28.5%; CV (block) = 12.4%

At GS25, the amount of *M. nivale* was reduced significantly by both seed treatments across the seed lots. Beret Gold reduced the amount of *M. nivale* significantly ($P < 0.05$) compared to Sibutol (Figure 7.23). There was no significant ($P < 0.05$) difference in the amount of *M. nivale* in seedlings produced from seed lots 1 and 2 though the amount was significantly ($P < 0.05$) less than in seedlings produced from seed lots 3 and 4, between which there was no significant ($P < 0.05$) difference. The amount of *M. nivale* was reduced significantly ($P < 0.05$) by both Sibutol and Beret Gold for seed lot 1 and 2. Beret Gold reduced the amount of *M. nivale* significantly ($P < 0.05$) compared to Sibutol for all four seed lots though there was no significant ($P > 0.05$) difference between Beret Gold treated and untreated for seed lot 3 or Sibutol and the untreated for seed lot 4.

At GS12, *Fusarium* spp. were detected in seedling samples from all plots, both seed treatments reduced the amount of *Fusarium* spp. significantly ($P < 0.05$) compared to the untreated across the seed lots (Figure 7.24). The amount of *Fusarium* spp. detected in the four seed lots was significantly ($P < 0.05$) different in all cases. Seedlings from seed lot 3 contained the least *Fusarium* spp. followed by 1, 4 and 2, respectively. Both seed treatments reduced the amount of *Fusarium* spp. significantly ($P < 0.05$) in seedlings produced from all four seed lots and Beret Gold reduced the amount of *Fusarium* spp. significantly ($P < 0.05$) compared to Sibutol for seed lots 1 and 3. At GS25 and 39, *Fusarium* spp. were not detected in any seedlings samples from the field trial plots.

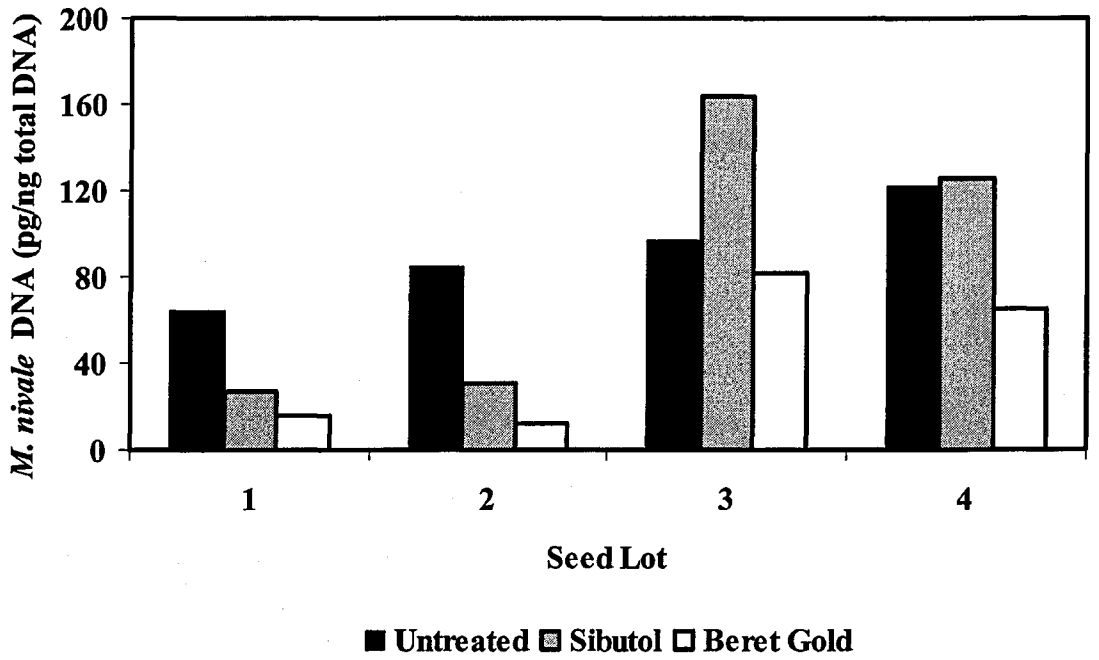


Figure 7.23. Effect of seed treatments on the quantity of *M.* nivale DNA in wheat seedlings cv. Equinox at growth stage 25 produced from separate seed lots in 1999. (see Table 7.12 for statistical analysis).

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

Table 7.12. Statistical analysis of *M. nivale* DNA data following Log transformation.

Treatment	Log *M. nivale* DNA / Seed lot				
	1	2	3	4	All
Untreated	1.76	1.92	1.99	2.08	1.94
Sibutol	1.31	1.31	2.15	2.08	1.72
Beret Gold	0.97	0.92	1.90	1.80	1.40
All	1.35	1.38	2.01	1.99	

N.B. *M. nivale* DNA data was Log transformed in order to normalise data prior to statistical analysis.

Seed Lot; SEM = 0.07; LSD = 0.15; $P = <0.001$
Treatment; SEM = 0.06; LSD = 0.13; $P = <0.001$
Treatment*Seed Lot; SEM = 0.13; LSD = 0.25; $P = <0.001$
D of F = 143; CV = 18.5%; CV (block) = 8.0%

Figure 7.24. Effect of seed treatments on the quantity of *Fusarium* spp. DNA in wheat seedlings cv. Equinox at growth stage 12 produced from separate seed lots in 1999. (see Table 7.13 for statistical analysis).

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

Table 7.13. Statistical analysis of *Fusarium* DNA data following Log transformation.

Treatment	Log *Fusarium* DNA / Seed lot				
	1	2	3	4	All
Untreated	-1.89	0.66	-2.14	0.32	-0.76
Sibutol	-3.10	-2.04	-2.93	-2.35	-2.60
Beret Gold	-2.42	-2.18	-3.88	-2.34	-2.71
All	-2.47	-1.19	-2.98	-1.46	

N.B. *Fusarium* spp. DNA data was Log transformed in order to normalise data prior to statistical analysis.

Seed Lot; SEM = 0.127; LSD = 0.251; P = <0.001
Treatment; SEM = 0.110; LSD = 0.217; P = <0.001
Treatment*Seed Lot; SEM = 0.219; LSD = 0.434; P = <0.001
D of F = 143; CV = 26.5%; CV (block) = 11.2%

Quantification of M. nivale *var.* nivale *and* M. nivale, *var.* majus *in seedlings from 1999 trial*

At GS12, neither *M. nivale* var. *nivale* or var. *majus* could be detected in Beret Gold treated seedlings from seed lots 2, 3 or 4 or in any seedlings produced from seed lot 1 irrespective of treatment. Seedlings produced from untreated seed lots 2, 3 and 4 contained the most *M. nivale* var. *majus* and those produced from untereated seed lot 4 contained the most *M. nivale var. nivale.* Sibutol reduced the amount of both *M. nivale* var. *nivale* and var. *majus* significantly ($P < 0.05$) compared to the untreated across the seed lots (Figure 7.25).

At GS25, *M. nivale* var. *nivale* and var. *majus* were detected in seedlings sampled from all plots. The amount of *M. nivale* var. *majus* was reduced significantly ($P < 0.05$) by both Sibutol and Beret Gold compared to the untreated across the four seed lots and Beret Gold reduced the amount of *M. nivale* var. *majus* in seedlings significantly ($P < 0.05$) more than Sibutol (Figure 7.26).

The amount of *M. nivale* var. *nivale* detected in seedlings produced from Sibutol treated seed from seed lots 3 and 4 was significantly ($P < 0.05$) greater than that detecetd in seedlings produced from the respective untreated seed. Beret Gold reduced the amount of *M. nivale* var. *nivale* in seedlings at GS25 significantly ($P < 0.05$) across the four seed lots, compared to Sibutol treated and untreated seed.

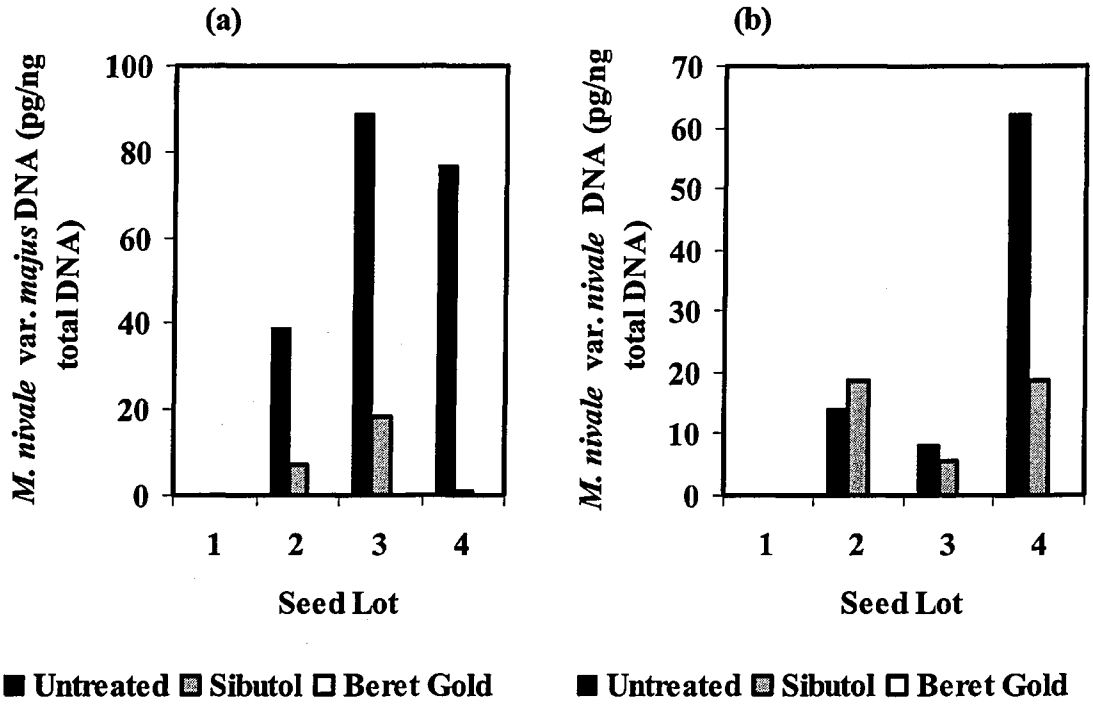


Figure 7.25. Effect of seed treatments on the quantity of (a) *M. nivale* var. *majus* and (b) *M. nivale* var. *nivale* DNA in wheat seedlings cv. Equinox at growth stage 12 grown from four separate seed lots in 1999. (see Table 7.14 for statistical analysis).

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

Table 7.14. Statistical analysis of *M. nivale* var. *majus* and var. *nivale* DNA data following Log (DNA+1) transformation, ND = Not Detected.

Treatment	Log (*M. nivale* var. *majus* DNA +1)					Log (*M. nivale* var. *nivale* DNA +1)				
	1	2	3	4	All	1	2	3	4	All
Untreated	ND	1.23	1.93	1.74	1.63	ND	0.82	0.87	1.41	1.04
Sibutol	ND	0.74	1.06	0.27	0.69	ND	0.56	0.76	0.85	0.72
Beret Gold	ND	ND	ND	ND	-	ND	ND	ND	ND	-
All	-	0.98	1.50	1.01		-	0.69	0.82	1.13	

N.B. Transformation; Log (*M. nivale* DNA +1) was used in order to normalise data prior to statistical analysis.

M. nivale var. *majus*
Seed Lot; SEM = 0.16; LSD ($P = 0.05$) = 0.32; $P = 0.004$
Treatment; SEM = 0.13; LSD ($P = 0.05$) = 0.26; $P = <0.001$
Treatment*Seed Lot; SEM = 0.16; LSD ($P = 0.05$) = 0.45; $P = 0.01$
D of F = 47; CV = 39.0%; CV (block) = 11.5%
M. nivale var. *nivale*
Seed Lot; SEM = 0.17; LSD ($P = 0.05$) = 0.35; $P = 0.04$
Treatment; SEM = 0.14; LSD ($P = 0.05$) = 0.28; $P = 0.03$
Treatment*Seed Lot; SEM = 0.24; LSD ($P = 0.05$) = 0.49; $P = 0.42$
D of F = 47; CV = 55.1%; CV (block) = 16.1%

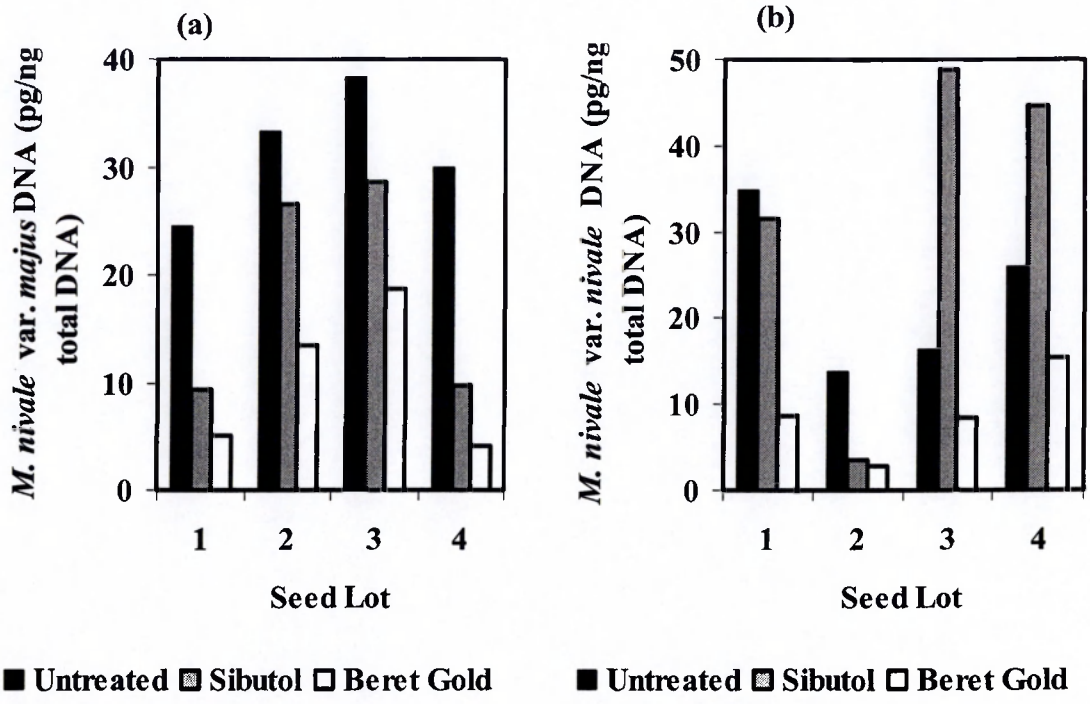


Figure 7.26. Effect of seed treatments on the quantity of (a) *M. nivale* var. *majus* and (b) *M. nivale* var. *nivale* DNA in wheat seedlings cv. Equinox at growth stage 25 grown from four separate seed lots in 1999. (see Table 7.15 for statistical analysis).

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

Table 7.15. Statistical analysis of *M. nivale* var. *majus* and *nivale* DNA data following Log (DNA+1) transformation, ND = Not Detected.

Treatment	Log *M. nivale* var. *majus*					Log *M. nivale* var. *nivale*				
	1	2	3	4	All	1	2	3	4	All
Untreated	1.24	1.48	1.59	1.43	1.44	1.26	1.06	1.22	1.42	1.24
Sibutol	0.91	1.39	1.39	0.95	1.16	0.79	0.61	1.66	1.60	1.16
Beret Gold	0.74	1.13	1.28	0.71	0.96	0.88	0.50	0.91	1.13	0.85
All	0.96	1.33	1.42	1.03		0.97	0.72	1.26	1.38	

N.B. *M. nivale* var.*inivale* and var. *majus* DNA data was Log transformed in order to normalise data prior to statistical analysis.

M. nivale var. *majus*
Seed Lot; SEM = 0.07; LSD (P = 0.05) = 0.14; P = <0.001
Treatment; SEM = 0.06; LSD (P = 0.05) = 0.12; P = <0.001
Treatment*Seed Lot; SEM = 0.12; LSD (P = 0.05) = 0.23; P = 0.15
D of F = 95; CV = 19.8%; CV (block) = 6.6%

M. nivale var. *nivale*
Seed Lot; SEM = 0.10; LSD (P = 0.05) = 0.21; P = <0.001
Treatment; SEM = 0.09; LSD (P = 0.05) = 0.18; P = <0.001
Treatment*Seed Lot; SEM = 0.18; LSD (P = 0.05) = 0.36; P = 0.003
D of F = 95; CV = 33.1%; CV (block) = 15.4%

At GS59, the amount of *M. nivale* var. *majus* detected in plants produced from Sibutol and Beret Gold treated seed was significantly ($P < 0.05$) greater than the amount detected in plants produced from untreated seed across the seed lots (Figure 7.27). Significantly ($P < 0.05$) more *M. nivale* var. *majus* was detected in plants produced from seed lot 1 than those produced from the other three seed lots across the treatments. Sibutol treated seed from seed lot 1 produced plants which contained significantly ($P < 0.05$) more *M. nivale* var. *majus* than those from any of the other eleven treatments in the trial. In general, Sibutol and Beret Gold treated seed produced plants which contained more *M. nivale* var. *majus* than those produced from the respective untreated seed. The amount of *M. nivale* var. *nivale* detected in plants produced from Beret Gold was significantly lower ($P < 0.05$) than that detected in plants produced from either untreated or Sibutol treated seed across the seed lots. Plants produced from seed lot 1 contained significantly ($P < 0.05$) less *M. nivale* var. *nivale* than those produced from the other three seed lots and plants produced from seed lot 4 contained the greatest amount of *M. nivale* var. *nivale.*

Relationship between total M. nivale *var.* nivale *and var.* majus *and total* M. nivale *quantification*

The relationship between the quantification of total *M. nivale* using the JBM quantitative PCR assay and total *M. nivale* determined using the total of *M. nivale* var. *nivale* and var. *majus* using the sub-species specific quantitative PCR assays (Chapter 3) in seedlings produced at GS25 showed a highly significant ($P < 0.001$) relationship (Figure 7.28). Two significant outliers were identified in the data set, this could have been due to experimental error. Following the removal of the outliers from the data set, the equation of the regression line was $y = 1.95x + 3.93$ and the R^2 for the relationship was 0.78.

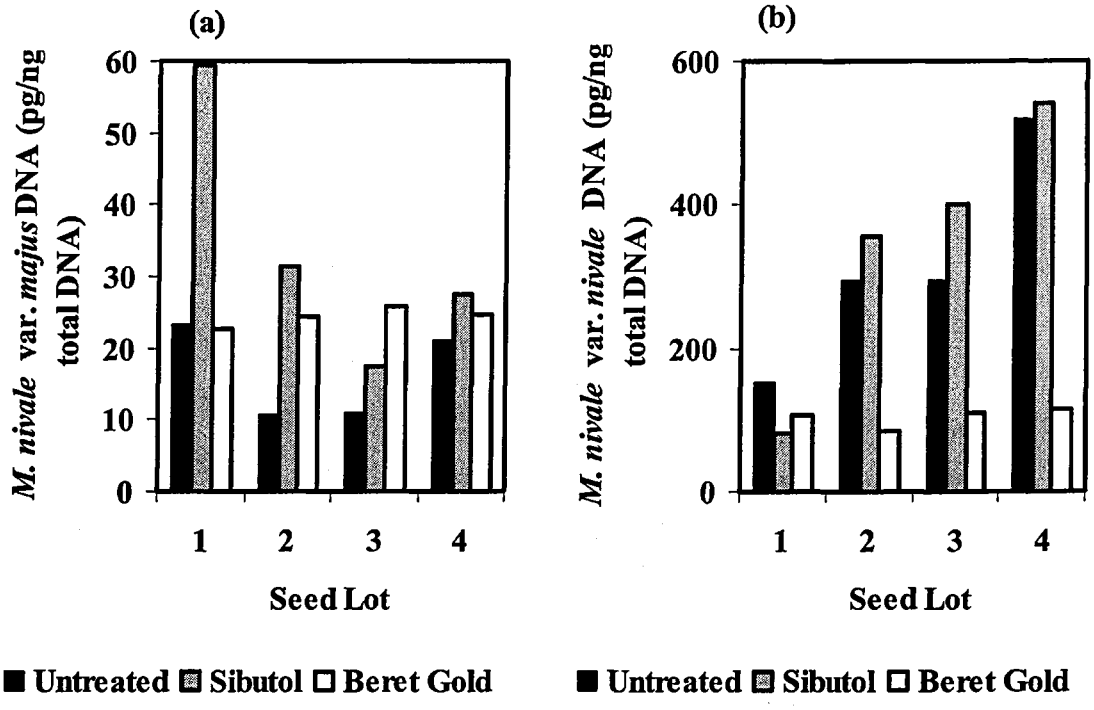


Figure 7.27. Effect of seed treatments on the quantity of (a) *M. nivale* var. *majus* and (b) *M. nivale* var. *nivale* DNA in wheat plants cv. Equinox at growth stage 59 grown from four separate seed lots in 1999. (see Table 7.16 for statistical analysis).

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

Table 7.16. Statistical analysis of *M. nivale* var. *majus* and var. *nivale* DNA data following Log transformation. ND = Not Detected.

Treatment	*M. nivale* var. *majus*					*M. nivale* var. *nivale*				
	1	2	3	4	All	1	2	3	4	All
Untreated	1.34	1.04	1.05	1.30	1.18	2.12	2.45	2.42	2.69	2.42
Sibutol	1.73	1.49	1.18	1.43	1.46	1.83	2.53	2.58	2.68	2.40
Beret Gold	1.36	1.40	1.39	1.34	1.37	1.98	1.85	2.01	2.03	1.97
All	1.48	1.31	1.20	1.36		1.97	2.27	2.34	2.47	

N.B. *M. nivale* var.*inivale* and var. *majus* DNA data was Log transformed in order to normalise data prior to statistical analysis.

M. nivale var. *majus*
Seed Lot; SEM = 0.05; LSD ($P = 0.05$) = 0.11; $P = <0.001$
Treatment; SEM = 0.05; LSD ($P = 0.05$) = 0.09; $P = <0.001$
Treatment*Seed Lot; SEM = 0.09; LSD ($P = 0.05$) = 0.18; $P = <0.001$
D of F = 95; CV = 13.7%; CV (block) = 5.8%

M. nivale var. *nivale*
Seed Lot; SEM = 0.06; LSD ($P = 0.05$) = 0.12; $P = <0.001$
Treatment; SEM = 0.05; LSD ($P = 0.05$) = 0.11; $P = <0.001$
Treatment*Seed Lot; SEM = 0.11; LSD ($P = 0.05$) = 0.22; $P = <0.001$
D of F = 95; CV = 9.6%; CV (block) = 2.4%.

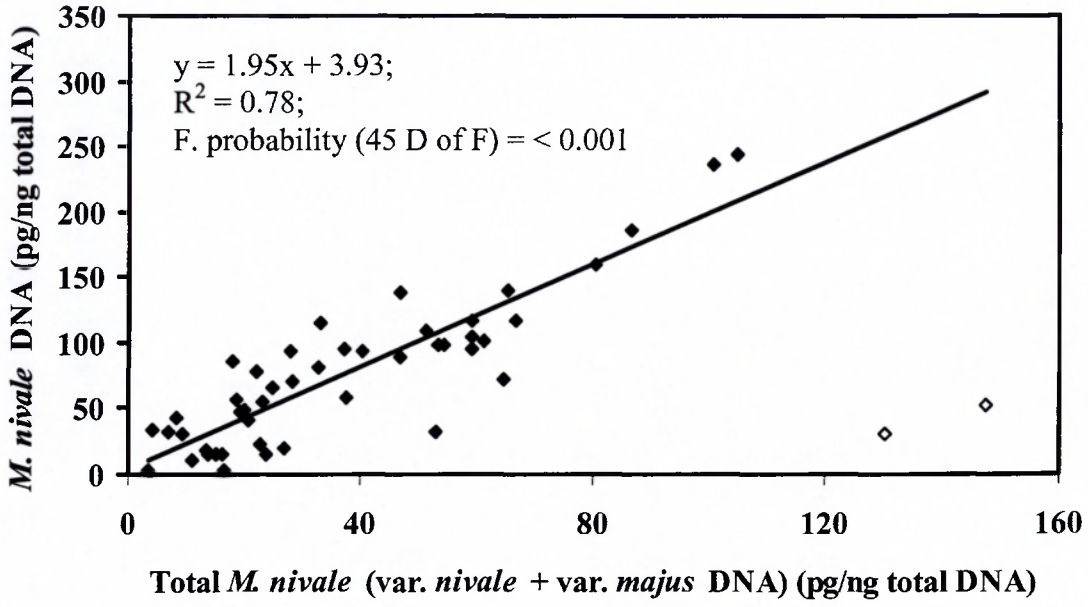


Figure 7.28. Relationship between the quantification of total *M. nivale* DNA using the JBM quantitative PCR assay (y axis) and total *M. nivale* DNA determined as *M. nivale* var. *nivale* DNA + var. *majus* DNA (x axis) determined using the sub-species specific quantitative PCR assays based on the elongation factor 1α (Chapter 3) in wheat seedlings cv. Equinox at growth stage 25.

Ratio between M. nivale *var.* nivale *and var.* majus *in 1999 field trial*

As no *M. nivale* var. *nivale* or var. *majus* could be detected in any seedlings produced from seed lot 1 or any seedlings produced from Beret Gold treated seed, no pathogen ratios for these treatments could be determined. At GS12, pathogen ratios were significantly ($P < 0.05$) greater for seedlings produced from Sibutol treated seed than for those produced from untreated seed across the seed lots (Figure 7.29). No significant ($P < 0.05$) difference was observed between the seed lots or between treatment and seed lot. At GS25, no significant ($P > 0.05$) difference was observed between pathogen ratios for the three treatments across the seed lots (Figure 7.29). At GS59, pathogen ratios were significantly ($P < 0.05$) lower for seedlings produced from Sibutol and Beret Gold treated seed than for those produced from untreated seed across the seed lots indicating that *M. nivale* var. *nivale* was the predominant sub-species present (Figure 7.29). Beret Gold treated seed produced plants which showed significantly ($P < 0.05$) lower pathogen ratios than those produced from Sibutol treated seed.

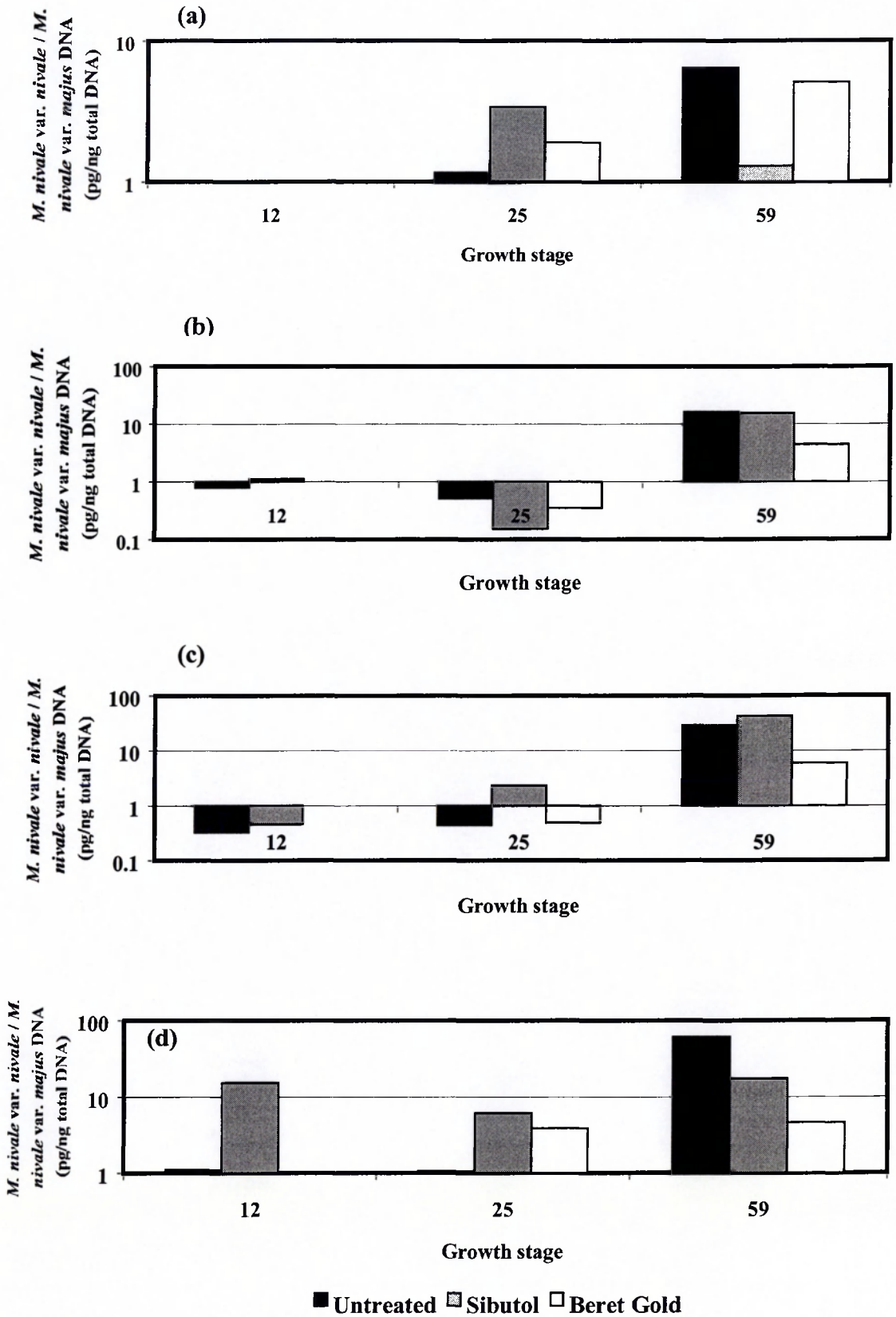


Figure 7.29. Ratio of *M. nivale* var. *nivale* DNA : var. *majus* DNA detected in emerged wheat plants cv. Equinox produced from four separate seed lots in 1999 (a) Seed Lot 1, (b) Seed Lot 2, (c) Seed Lot 3 and (d) Seed Lot 4. No data available for GS12 for seed lot 1 or Beret Gold treated seed lots 2, 3 and 4.

Key to seed lots; seed lot and pathogen used to inoculate seed production plots at anthesis in **1999**; seed lot 1 = Commercial seed; seed lot 2 = *F. culmorum*; seed lot 3 = *M. nivale* var. *majus*; seed lot 4 = *M. nivale* var. *nivale*.

DISCUSSION

Of the six fungicide seed treatments tested, isolates of *M. nivale* var. *nivale* and var. *majus* were most sensitive to Beret Gold which was more active against the pathogen than the least active fungicide (Anchor). Isolates of *M. nivale* var. *majus* (with the exception of the outlier R17Db) showed less variability in sensitivity towards Beret Gold than isolates of var. *nivale*. Isolate R17Db showed reduced sensitivity towards Beret Gold compared to other isolates of *M. nivale* assigned to the var. *majus* group. This may be a result of mis-identification of this isolate as a member of the var. *majus* sub-species or genuinely reduced sensitivity towards the active ingredient, the phenylpyrrole fungicide fludioxonil. DNA isolated from cultures of this isolate amplified using *M. nivale* var. *nivale* primers and not *M. nivale* var. *nivale* specific primers. Attempts to produce spores of this isolate following the fungicide sensitivity experiments failed despite spores of the isolate being readily produced in initial isolations. This further confirms this isolate as a significant out group compared to the other *M. nivale* isolates tested. Significant outliers in sensitivity towards Baytan whose active ingredients are fuberidazole (MBC fungicide) and triadimenol (triazole fungicide) within the var. *nivale* and var. *majus* isolates were also observed. In a survey of MBC resistance in *Fusarium* spp., Locke *et al.* (1987) reported that 92.1% of 581 isolates of *M. nivale* were able to grow on PDA amended with 20 mg l^{-1} MBC, such isolates were termed by the authors as resistant to the fungicide. Isolates of *M. nivale* unable to grow on 20 mg l^{-1} MBC were termed sensitive to the fungicide by Locke *et al.* (1987). All isolates used in this study would therefore have been classified by Locke *et al.* (1987) as sensitive to MBC fungicides. This may be a result of increased activity of triadimenol towards the pathogen or an increase in sensitivity towards MBC fungicides since the study by Locke *et al.* (1987) due to their reduced use as fungicide sprays. In this experiment, seed treatment preparations were used as opposed to the pure active ingredient used by Locke *et al.* (1987). Thus, compounds present in the seed treatment formulation may have enhanced the activity of the active ingredients. Hilber (1992) identified

phenylpyrrole fungicides as being of high resistance risk as they are applied as single active ingredients and strains of *Botrytis cinerea* which were highly resistant to fludioxonil were readily produced under laboratory conditions. Glynn (unpublished data) detected reduced sensitivity towards fludioxonil in mutant isolates of *M. nivale* produced through either UV irradiation of spores or exposure to the fungicide. Mutant isolates exhibiting reduced sensitivity towards fludioxonil had EC_{50} values between 0.4 mg l^{-1} and >6.4 mg l^{-1} compared to the wild type strain sensitivity of 0.1 mg l^{-1}. Reduced sensitivity towards fludioxonil was similar to that observed in sensitivity to the MBC fungicide benomyl and took place in a single step mutation. All mutants exhibiting reduced sensitivity towards fludioxonil were, however, highly sensitive to increased concentrations of glucose *in vitro*, a characteristic indicative of reduced fitness (Hilber, 1992). The experiments of Glynn (unpublished) and the present investigation are not directly comparable as the former study used active ingredients. However, the reduced sensitivity of R17Db or the difference in sensitivity towards fludioxonil shown by the two sub-species of *M. nivale* is unlikely to be a result of mutation or selection towards resistance. If this had been the case, a much larger increase in sensitivity would be expected following a single step-gene mutation as was reported previously by Glynn (unpublished data) and in *B. cinerea* by Hilber (1992). Thus the reduced sensitivity of R17Db and all isolates of *M. nivale* var. *nivale* compared to the remaining isolates of *M. nivale* var. *majus* is likely to be due to physiological or metabolic polymorphisms inherent in those isolates tested rather than selection or mutation towards resistance. The sensitivity of the *M. nivale* isolates tested towards Sibutol did not differ between the two sub-species. The major component of Sibutol is the triazole fungicide bitertanol. Reduced sensitivity towards triazole fungicides is polygenic and thus a population shift towards reduced sensitivity would be expected as isolates expressing several resistance genes are selected under fungicide pressure (Brent, 1995). Several triazole fungicides are currently available for the control of fusarium diseases such as

epoxiconazole, tebuconazole, triticonazole, metconazole and fluquinconazole (Anon, 2001). These fungicides have similar modes of action (ergosterol biosynthesis inhibition), therefore, reduced sensitivity to one product may lead to reductions in sensitivity towards other members of the triazole group. Through their widespread use, the selection pressure on populations of fusarium disease causing pathogens is likely to be high. An annual monitoring programme of sensitivity towards triazole fungicides in field isolates of *M. nivale* would be the most effective way of determining any shifts in population sensitivity towards triazole fungicides. A monitoring of sensitivity towards phenylpyrrole fungicides would also require determination of the fitness parameters in any strain less sensitive than sensitive wild-type strains, for example sensitivity to osmotic pressure.

In vitro fungicide sensitivity experiments such as the ones performed in this study give an indication as to the activity of fungicidal products against pathogens which are responsible for the disease concerned. Such experiments however, provide only limited information as to the likely field performance of the test product as factors such as the mobility and persistence of the active ingredients within and around the host plant affect the overall efficacy of the fungicide product. In the case of Anchor, which in this study showed least activity towards *M. nivale*, a possible mode of action of one of its active ingredients (carboxin) has been reported as being by the induction of pathogenesis related proteins in the host (Chilosi *et al.*, 2001). In the USA, carboxin is registered as a plant growth regulator thus disease control may be achieved through a combination of plant induced response rather than a direct effect on the pathogen responsible for the disease. Such indirect activity is not detected within an *in vitro* assay.

Controlled environment studies allow the performance of seed treatments to be assessed under specific environmental conditions. In this case, temperature which has a profound affect on the expression of seedling blight disease was chosen in order to assess the robustness of seedling blight control. The greatest improvements in emergence due to

the seed treatments used were evident at 4°C where emergence was lowest in the untreated. Few differences in the emergence of treated and untreated seedlings were evident as the temperature was increased to 16°C. Hare (1997) reported that the emergence of wheat seedlings from *M. nivale* infected seed was significantly reduced at 6°C compared to 8°C, 10°C and 12°C. Humphreys *et al.* (1995) reported that following severe winter kill of wheat seedlings by *M. nivale*, those surviving were largely free from infection. This may account for the reduced incidence and severity of stem-base symptoms on seedlings grown at 4°C which had the lowest emergence compared to those grown at 8°C and 12°C which showed higher emergence scores and a greater incidence and severity of stem-base disease symptoms. Previous experiments in this study (Chapter 4) have shown the opposite effect; reduced emergence correlating to increased stem-base disease incidence and severity. The mean temperature over the course of that experiment was 7.5°C. Had the experiment been performed at a lower temperature however, emerged seedlings may have had lower amounts of stem-base disease. Reduced temperatures favour severe infections caused by *M. nivale*, the most severe being pre-emergence damping off. At temperatures of 8°C and 12°C, both the pathogen and plant are able to develop, at 16°C plant growth was favoured and disease symptoms though still observed were much less severe. All treatments were robust with respect to improvements in emergence and performed equally well when the reduction in emergence was greatest at 4°C. The results from the *in vitro* sensitivity study (Beret Gold showing greater efficacy than Sibutol towards both sub-species) appeared to reflect most accurately the effect on stem-base disease incidence and severity rather than the effect on seedling emergence. Sibutol was less robust than either Beret Gold or Celest Extra with respect to the incidence and severity of stem-base disease symptoms when disease pressure was high (at 8°C and 12°C). In growth room experiments, West *et al.* (2001) reported that fludioxonil improved the emergence of seedlings produced from *M. nivale* infected seed (49% infection) and also the percentage of healthy seedlings compared

to three triazole fungicide seed treatments. Unfortunately, the three fungicides used in the experiments performed by West *et al.* (2001) did not include those used in the present study. When disease pressure from *M. nivale* var. *nivale* was high (at 8°C) Beret Gold performed less well than Celest Extra with respect to stem-base disease incidence and severity. The active ingredient present in Beret Gold (fludioxonil) is also present in Celest Extra at the same rate (50 g l^{-1}), however, the latter seed treatment also contains the triazole fungicide difenoconazole (A8179b). The isolates of *M. nivale* used in this study showed reduced sensitivity towards Celest Extra compared to Beret Gold during *in vitro* experiments. It would appear, therefore, that a synergistic or additive effect between fludioxonil and difenoconazole may exist with respect to stem-base disease caused by *M. nivale* var. *nivale* the result being improved reductions of stem-base disease incidence and severity.

The difference in sensitivity between *M. nivale* var. *nivale* and var. *majus* towards Beret Gold observed in *in vitro* experiments was not evident in the controlled environment experiments. This suggests that the difference to the previous experiments are a result of differential interaction with the media used (PDA) with respect to fungicide uptake or translocation *in vitro* or a similar effect in whole plant experiments. Alternatively, the small differences observed during the *in vitro* studies may not be readily detected in whole plant studies.

The artificial inoculation of crops is often used in fungicide efficacy trials so the performance of fungicides can be determined under high disease pressure. Inoculated wheat plots which were mist irrigated allowed the production of infected grain for use in fungicide seed treatment trials. However, natural inoculum present within the crop was able to develop more extensively than under normal field conditions. This, combined with the possible spread of inoculum between inoculated plots may have accounted for the detection of *Fusarium* spp. in seed from *M. nivale* inoculated plots and *M. nivale* var.

nivale in seed from var. *majus* inoculated plots and vice versa in both years. Ear infections of wheat by *F. graminearum* and *F. culmorum* are favoured by temperatures in the range 20 - 30°C (Sutton, 1982) and are optimal around 25°C (Andersen, 1948, Parry *et al.*, 1994). In the case of *F. graminearum* however, negligible head infection has been reported at 15 °C (Andersen, 1948) whereas for *F. culmorum*, little difference was observed in the percentage of florets infected by *F. culmorum* at 15°C and 20°C (Parry *et al.*, 1994). The mean maximum daily temperature during the mist irrigation process in this experiment was only 18.2°C (maximum 24.4°C, minimum 13.2°C) which may explain the limited infection by *F. graminearum*. No information is available on differential temperature requirements for head infections caused by the two *M. nivale* sub-species. The incidence of seed infection by the two *M. nivale* sub-species was similar for the two seed lots which were produced from field plots inoculated with var. *nivale* and var. *majus* separately, however, more *M. nivale* var. *nivale* was detected in these seed lots using the quantitative PCR assays. This suggests either that var. *nivale* caused more extensive infections of individual grains in the developing ear or that more *M. nivale* var. *nivale* than var. *majus* was present on the seed surface, a trend which corroborates the findings of Chapter 4 using commercial grain samples.

Germination tests suggested that the ability of *M. nivale* to inhibit the germination of infected seed is dependent upon temperature germination of untreated, predominantly *M. nivale*-infected seed was lower at 4°C than at 18°C. This does not appear to be the case for *F. culmorum* where low germination was evident at both of the temperatures employed. This could be a result of more dead seed being present in the *F. culmorum* infected seed as a result of severe ear infection. The stand count results for the *Fusarium* spp. infected seed at GS25 were lower than those for the predominantly *M. nivale* infected seed batches in both years suggesting that *Fusarium* spp. were more aggressive than *M. nivale* as a cause of pre-emergence damping off. This result contradicts the findings of earlier experiments

(Chapter 4) where seed infection by *M. nivale* was more closely associated with pre-emergence damping off than *Fusarium* spp. Similar stand counts were observed from the predominantly *Fusarium* spp. infected seed as the predominantly *M. nivale* infected seed. This occurred despite more *Fusarium* spp. than *M. nivale* being detected in those seed batches by either plate counts or quantitative PCR. This suggests that *M. nivale* as a cause of pre-emergence damping off is more pathogenic than *Fusarium* spp. 'per unit of pathogen detected'.

The amount of pathogen in each seed lot was reflected in the amount of pathogen detected in seedlings from untreated seed. In both years, those seed lots that contained the most *M. nivale* or *Fusarium* spp. produced seedlings infected with the most *M. nivale* or *Fusarium* spp. respectively. More *M. nivale* was detected in seedlings in 1999 than in 1998, and more *Fusarium* was detected in untreated seedlings from the *F. culmorum* infected seed (lot 2) in 1998 than in 1999 despite more *Fusarium* being detected in the seed lot in the latter year. This could be a result of separate environmental conditions which favoured infection by each group of pathogens (Colhoun *et al.*, 1968, Millar and Colhoun, 1969, Parry *et al.*, 1994). The mean daily soil temperature between drilling and sampling for the 1998 trial was 12.2°C (minimum 8.0°C maximum 14.6°C) whereas for the 1999 trial the mean soil temperature was 9.5°C (minimum 6.5°C maximum 11.7°C) between drilling and sampling. The lower soil temperature in the 1999 trial may account for the higher level of infection by *M. nivale* compared to *Fusarium* spp. in terms of reduced emergence and increased pathogen DNA respectively.

Despite the detection of more *Fusarium* spp. DNA in the *F. culmorum* inoculated seed in 1999 than in 1998, seedlings produced in 1998 contained more *Fusarium* spp. than in 1999. *Fusarium* spp. were detected in all seedling samples in 1999 but only seedlings from seed lot 2 in 1998. Conditions may have been more conducive to infection from *Fusarium* spp. in the 1999 trial, or the increased infection could be a result of different

varieties being used even though they do have similar Fusarium ear blight resistance ratings (Anon, 1999). A further explanation may be that when severe disease caused by *M. nivale* occurs, as was the case in the 1999 trial, infected seedlings are more susceptible to infection by seed-borne *Fusarium* spp. In the 1998 trial, *Fusarium* spp. only caused severe infections when present in the seed in the greatest amounts. Millar and Colhoun (1969) stated that when applied to the seed surface, high spore loads of *F. culmorum* could act as a substitute for unfavourable environmental conditions in determining seedling disease. Whereas, Colhoun *et al.* (1968) reported that under favourable environmental conditions, *M. nivale* could cause severe infection even if the number of spores per seed was extremely low. In this investigation, the amount of *Fusarium* spp. detected in the eight seed lots was much greater than *M. nivale* though in general, more *M. nivale* than *Fusarium* spp. was detected in infected seedlings except when *Fusarium* spp. were present in the seed at the highest level. The conclusions of the earlier workers, based on the number of spores artificially applied to the surface of wheat seeds may be corroborated by results from this study where the amount of pathogen inoculum contained within a sample of seed has been quantified. However, neither of the previous studies considered interactions between *Fusarium* spp. and *M. nivale* in relation to seedling disease. In 1998, the lowest emergence was observed from seed which contained the greatest amount of *Fusarium* spp. whereas seed with the greatest amount of *M. nivale* had significantly higher emergence. In 1999, no significant difference was observed in emergence between the untreated seed for the three infected seed lots which were produced. This suggests that the ability of *F. culmorum* to cause pre-emergence seedling death was affected less by temperature than *M. nivale* or again that the reduction in emergence caused by infection from *F. culmorum* was due to the pathogen killing seeds as a result of severe ear infections. Infection from soil-borne pathogens would seem unlikely as the amount of pathogen DNA detected in the

untreated seedlings produced from the commercial seed, which contained the least amount of pathogen in the seed, was low and stand counts were high in both years.

Beret Gold (a.i. fludioxonil) and Sibutol (a.i.'s bitertanol + fuberidazole) were chosen in this investigation as *in vitro* tests had identified them as being the most active fungicide seed treatments against isolates of *M. nivale*. Generally, fludioxonil showed better performance than bitertanol + fuberidazole towards *M. nivale*, this was significantly better when the pressure from infection was particularly high in 1999. The level of control for the *F. culmorum* infected seed was high (90%) for both fungicides. In field trials conducted in Great Britain in 1992, Koch and Leadbeater (1992) recorded a mean stand improvement for winter wheat of 172%, 37-56 days after drilling using fludioxonil as a seed treatment and seed with 70% infection of *M. nivale*. They recorded a mean stand increase of 195.5% at the second leaf stage (GS12) in field trials conducted in France and Switzerland using fludioxonil and seed with a high incidence of *F. culmorum* (90-100%). Using bitertanol + fuberidazole, Morris *et al.* (1994) recorded mean stand counts of 226% and 217% relative to the untreated controls using wheat cv. Haven or cv. Riband respectively and achieved reductions in stem base symptoms of 87% and 84% respectively. Morris *et al.* (1994) did not comment on the incidence or severity of seed infection and neither Koch and Leadbeater (1992) nor Morris *et al.* (1994) commented on the amount of cross contamination from other seedling blight pathogens. Unfortunately, neither set of experiments involved comparisons between the two seed treatments used in our study. No significant improvement in emergence was apparent in either field trial for the commercial seed with low infection. This is not unexpected as working with treated and untreated seed with low *M. nivale* infection, Paveley and Davies (1994) found no benefit from seed treatments when seed was sown at a number of field sites in the UK.

Humphreys *et al.* (1995) reported that in field studies and in trays of compost, seedlings that survived pre-emergence mortality caused by *M. nivale* or *Fusarium* spp.

were largely free from infection following establishment. Jones (1999) reported that the dry weight of seedlings which emerged from seed with a high incidence of *F. graminearum* (70%) was equivalent to that for seedlings produced from seed with a low incidence (2%) of the pathogen. Unfortunately, Jones did not comment on the symptoms or state whether the pathogen could be detected in surviving plants. Hare (1997) reported reductions in emergence of 70% due to seed borne *M. nivale* in experiments performed at 6°C; all surviving seedlings showed stem-base symptoms. When the same seed was sown at 12°C, emergence was reduced by only 20% and only 40% of surviving plants showed visual symptoms which were less severe. Severe symptoms were also reported on seedlings which survived pre- and post-emergence death caused by *F. graminearum* (Kane and Smiley, 1987). These results showed that treatments with the lowest emergence scores had higher pathogen DNA contents and are in keeping with the findings of both Hare (1997) and Kane *et al.* (1987). The contradiction to the findings of Humphreys *et al.* (1995) may be a result of the detection of both symptom-causing and symptom-less pathogen infections in this study.

In previous experiments (Chapter 5), the amount of *M. nivale* var. *nivale* detected in seed samples was more closely associated with stem-base disease than *M. nivale* var. *majus* which showed a closer correlation to reduced emergence. *M. nivale* var. *majus* was generally the predominant sub-species present during the early stages of crop development in the 1999 trial and as the season progressed var. *nivale* became the predominant sub-species detected which supports the idea of *M. nivale* var. *nivale* being a more aggressive coloniser of the stem-base than *M. nivale* var. *majus*. Alternatively, seedlings infected with *M. nivale* var. *majus* during the early stages of crop development may have been killed post-emergence due to the var. *majus* being more pathogenic than var. *nivale*. Seedlings surviving pre- and post- emergence death would therefore be infected predominantly with var. *nivale* which in the absence of var. *majus* and *Fusarium* spp. was

able to develop extensively within the stem-base. Sibutol generally performed less well than Beret Gold towards either sub-species particularly towards *M. nivale* var. *nivale*. At GS59, plants produced from seed treated with either seed treatment contained more *M. nivale* var. *majus* than from those produced from untreated seed, this may be a result of the predominance of var. *nivale* on untreated plants. Where a treatment was used and the amount of var. *nivale* reduced, *M. nivale* var. *majus* was able to develop more extensively than on untreated plants. West *et al.* (2001) reported that three triazole fungicides applied as seed treatments improved the emergence of *M. nivale* infected seed compared to the untreated. However, the three fungicides had no effect on the incidence of disease on emerged plants compared to the untreated. Fludioxonil however, showed large improvements in emergence and reductions in the incidence diseased plants. Unfortunatley, West *et al.* (2001) did not use the seed treatment used in this study (Sibutol).

CHAPTER 8

GENERAL DISCUSSION

GENERAL DISCUSSION

Microdochium nivale and several species of *Fusarium* can cause economically important diseases of UK wheat; seedling blight, foot rot and ear blight. The reduction in plant numbers caused by seedling blight can lead to reductions in grain yield and quality and provide a source of inoculum for Fusarium foot rot later in the season. Severe foot rot infections caused by *M. nivale* or *Fusarium* spp. may in turn lead to ear blight epidemics though the precise way in which this occurs is not fully understood. *Microdochium nivale* and *F. culmorum* are the primary causal agents of Fusarium seedling blight in the UK and of the two pathogens, *M. nivale* predominates. *Microdochium nivale* has long been recognised as consisting of isolates which are diverse in nature though conflicting evidence was reported as to the existence of sub-groups within the species before Lees *et al.* (1995) correlated phenotypic spore characteristics to RAPD profiles. They proposed that a largely homogenous sub-species existed within *M. nivale* which they termed variety *majus*, all other isolates, though somewhat more diverse were termed variety *nivale*. The trend they observed however, was not consistent among all the isolates which they tested. Nicholson *et al.* (1996a) developed primers and a quantitative assay for *M. nivale* var. *majus* and for var. *nivale* using the RAPD fragments generated by Lees *et al.* (1995) though the two assays were based on unknown genomic regions and therefore regions of unknown copy number.

The existence of sub-species within *M. nivale* led to questions relating to their relative pathogenicity, geographic distribution and fungicidal control; the development of quantitative PCR assays, in theory, facilitated the answering of these questions.

The development of quantitative PCR assays based on unknown genomic regions and regions of unknown copy number means that data relating to the relative amounts of two very closely related pathogens such as the two *M. nivale* sub-species is difficult to interpret.

In this investigation attempts were made to develop PCR primers for the two *M. nivale* sub-species which were based on the highly repeated rDNA which is conserved within many fungal species though heterologous to other fungal species. The high level of homology between isolates from the two sub-species however precluded the design of PCR primers which would allow discrimination between the two. This result served to confirm the close genetic relationship between *M. nivale* var. *nivale* and var. *majus*. The Elongation factor 1-α was identified as a potential region with sufficient genetic variability to allow discrimination between *M. nivale* var. *nivale* and var. *majus*. The use of degenerate primers allowed sequence analysis of the EF1- α for isolates of var. *majus* and var. *nivale* which ultimately facilitated the development of sub-species specific primers for the two *M. nivale* varieties. Testing of these putatively sub-species specific primers showed that they amplified DNA from isolates of *M. nivale* from Asia, Europe, North America and New Zealand. Comparison with the previously available primers showed that the two sets of primers were in agreement except for the primers developed for *M. nivale* var. *nivale* by Nicholson *et al.* (1996a) did not amplify DNA from isolate NRRL3289 which was shown to be pathogenic towards wheat. The sequence information obtained for the EF1- α gene for isolates of *M. nivale* var. *nivale* and var. *majus* allowed phylogenetic analysis between the two sub-species based on sequence information of a known, functional gene to be made for the first time. The isolates of *M. nivale* used, though few in number compared to those used in other phylogenetic studies, were obtained from a range of geographic locations within Northern Europe where *M. nivale* is a significant cereal pathogen. Isolates of the two sub-species were readily distinguished by phylogenetic analysis and *M. nivale* var. *nivale* showed slightly more genetic variability than the isolates of variety *majus* examined.

The quantitative PCR assays developed proved useful for the quantification of *M. nivale* inoculum in seed samples for which the incidence of *M. nivale* had also been

determined using the traditional agar plate count technique. A significant relationship was found between PCR quantification and agar plate count determination of the severity of seed batch infection for seed samples obtained over three years (1997 – 1999). The relationship between determinates of the severity of infection by *M. nivale* (PCR quantifications of *M. nivale* sub-species and agar plate counts) and seedling blight disease symptoms showed significant relationships between PCR quantification and reduced emergence and stem-base disease incidence and severity. PCR quantification accounted for a greater proportion of the variability in the seedling blight data sets than agar plate counts. A closer correlation was observed between the amount of *M. nivale* var. *majus* inoculum present in seed batches from 1999 and reduced seedling emergence than the amount of *M. nivale* var. *nivale* inoculum which was more closely related to stem-base disease infections. This result was corroborated further by studies where isolates of *M. nivale* var. *majus* were shown to be more pathogenic towards wheat cv. Cadenza than isolates of *M. nivale* var. *nivale.* This occurred both when spores were applied to the surface of the seeds used and when infected seed was produced under glasshouse conditions.

Though surveys have been performed over many years which describe the incidence and severity of seedling blight, foot rot and ear infections caused by species of *Fusarium* and *M. nivale*, little information was available as to the relative distributions of the two *M. nivale* sub-species within UK seed batches. The quantitative PCR assays for *M. nivale* var. *nivale* and var. *majus* developed in this study and the seed samples used allowed such information to be obtained. The two sub-species occurred most commonly together, rarely was one variety detected in the absence of the other in seed samples from either England or Scotland from the three years (1997-1999). A positive relationship between the amount of *M.* nivale var. *nivale* and var. *majus* inoculum present within the seed batches from each year was observed. *Microdochium nivale* var. *majus* predominated

in Scottish samples from each year whereas var. *nivale* was the predominant sub-species present in English samples in 1997 and 1998 and var. *majus* predominanted in English samples from 1999. Reference to the literature suggests that the predominance of var. *majus* in Scottish seed samples may be associated with stem-base infections caused by *T. acuformis*. No significant relationship between the amount of *M. nivale* inoculum present in seed batches and the amount of *Fusarium* spp. detected using the rDNA quantitative PCR assays was found. This result probably reflects the conclusions of previous workers who reported that *M. nivale* and *Fusarium* spp. have different environmental requirements for ear infection of wheat and also have a differing response to fungicides.

The control of Fusarium seedling blight of wheat using fungicide seed treatments has changed significantly since the introduction of the first treatments in the early 20th century. The withdrawal from use of organomerurial treatments in the 1980's led to the introduction of compounds from a range of fungicide chemistry. The *in vitro* response of fungicide seed treatments allows the activity of the fungicidal compound towards the pathogen in question to be determined. In this study, the activity of several seed treatments towards the two sub-species showed a varying range of sensitivities. Beret Gold was most active towards isolates of either sub-species and was significantly more active against isolates of variety *majus*. Controlled environment studies were performed in Chapter 7 using seed naturally infected with isolates *M. nivale* var. *nivale* or var. *majus* and the two most active fungicide seed treatments identified from the *in vitro* studies (Beret Gold and Sibutol). Results showed that Beret Gold was more effective than Sibutol at reducing the incidence and severity of stem-base Fusarium seedling blight, however no difference in emergence was observed between the two seed treatments. Quantitative PCR assays were used to determine the amount of *M. nivale* var. *nivale*, var. *majus* and *Fusarium* spp. in seed batches produced in two years prior to their use in fungicide seed treatment trials. The assays were again used to quantify the amount of pathogen inoculum in seedlings

produced from treated and untreated seed in order to determine the performance of two seed treatments (Beret Gold and Sibutol). As with the *in vitro* and controlled environment studies, Beret Gold was more active than Sibutol. The quantification of *M. nivale* var. *nivale* and var. *majus* at three stages throughout the growing season showed that variety *majus* was the dominant sub-species present in the early stages of crop development (GS12) however, at GS25 and GS59, variety *nivale* generally predominated.

FURTHER WORK

From the work presented, several areas would provide interesting areas for further research:-

i) The present study demonstrated evidence relating to the the phylogenetic distinctions between *M. nivale* var. *nivale* and var. *majus* based on a functional protein encoding gene. Further phylogenetic studies involving isolates from wide range of geographic locations and several more functional genes would allow the precise relatedness of isolates of *M. nivale* var. *nivale* and var. *majus* to be determined world-wide. This could be performed using the putatively universal primers for several protein encoding gene described by Carbone and Kohn (1999).

ii) Annual and regional differences in *M. nivale* var. *nivale* and var. *majus* inoculum present on seed batches from England and Scotland were observed in this study, a possible explanation for which is the two sub-species have different optimal environmental requirements as ear blight pathogens leading to differing amounts of seed-borne infection depending on conditions. A further explanation was that an interaction between *M. nivale* var. *majus* and *Tapesia* spp. on the stem-base of wheat plants led to an increase in inoculum causing increased ear and ultimately seed-borne infection. An interesting area for further investigation would be to determine the precise optimal environmental requirements of the two *M. nivale*

sub-species as ear blight pathogens of wheat and the precise interaction between *M. nivale* and *Tapesia* spp. Experiments would be performed under a range of closely controlled environmental conditions and a consistent amount of inoculum would be used to inoculate wheat plants at anthesis or at the stem-base, the amount of seed-borne inoculum would be quantified in the resultant seed using the quantitative PCR assays and related to the environmental conditions employed.

iii) Molecular markers specific to individual isolates of *M. nivale* var. *nivale* or var. *majus* such as AFLP's could be used to study the movement of isolates of each sub-species through the season in the field. In this way, the precise relationship between seed-borne, stem-base and ear infections caused by isolates of the two sub-species could be determined at a range of locations. This could be related to results obtained from controlled environment studies described in (ii) above.

iv) This study has demonstrated that *M. nivale* var. *majus* is more pathogenic as a seedling blight pathogen than var. *nivale* under the conditions employed. Further work could be focused on examination of the pathogenicity of the these two sub-species as seedling blight pathogens under a range of controlled environmental conditions and seed with a range of *M. nivale* infections to determine the relationship between *M. nivale* sub-species contamination, seedling blight disease and environmental conditions.

v) The high pressure placed upon the wild-type populations of *M. nivale* by the use of triazole fungicides could make necessary a fungicide resistance monitoring survey of the wild-type population. This would be performed by obtaining single spore isolates of the pathogen each year and determining their *in vitro* sensitivity. By relating each years results to the previous years, any shifts in sensitivity could be determined.

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APPENDICES

Appendix 1. Artificial media and buffers.

Artificial media

Potato dextrose agar composition (g l^{-1})

20	glucose
15	agar
4	potato infusion (from 200g of potatoes)

Synthetic Nutrient Agar (g l^{-1})

20	agar
1	potassium dihydrogen orthophosphate (Fisher Scientific, UK)
1	potassium nitrate (Fisher Scientific, UK)
0.5	magnesium sulphate (Fisher Scientific, UK)
0.5	potassium chloride (Fisher Scientific, UK)
0.2	glucose (Fisher Scientific, UK)
0.2	sucrose (Fisher Scientific, UK)

Potato Dextrose Broth composition (g l^{-1})

4	potato infusion (from 200g of potatoes)
20	Bacto dextrose

Buffers

CTAB extraction buffer (g l^{-1})

87.7	sodium chloride (Lancaster synthesis, Ltd., UK)
25.0	L-sorbose (Sigma)
10	polyvinylpoly-pyrrolidone (PVPP) (Sigma)
10	N-lauroylsarcosine sodium salt (Sigma)
8	n-Hexadecyltrimethyl ammonium bromide (CTAB) (Sigma)
8	ethylenediamine-tetraacetic acid (EDTA) (Sigma)

TE buffer

10mM	Tris Chloride (pH 7.4) (Sigma)
1mM	EDTA (pH 8.0) (Sigma)

TAE buffer

0.04M	Tris-acetate (BDH)
0.001M	EDTA

Chelex extraction buffer (g per 20ml SDW)

1	chelex 100 (imnodiacetic acid) (Sigma)
0.25	activated charcoal (Sigma)

Appendix 2. Isolates used in Chapters 6 and 7 of this study (isolates used in Chapter 3 given in Appendix 6 and 7).

Isolate code	Species/ sub-species	Origin	Chapter
Nr4/N	var. *nivale*	Shropshire England	6,7
+315/22	var. *nivale*	England	6, 7
SO28N	var. *nivale*	Angus, Scotland	6, 7
SO47N	var. *nivale*	Scotland	6
SO04N	var. *nivale*	Scotland	6, 7
117/2/N	var. *nivale*	Wye, England	6,7
74/1/N	var. *nivale*	N. Yorkshire, England	6,7
139/2/N	var. *nivale*	Dernford, England	6,7
18/1/N	var. *nivale*	Bardney, England	6
NL139M	var. *majus*	Netherlands	6
SO47M	var. *majus*	Scotland	6, 7
SO30M	var. *majus*	Scotland	6,7
24/3/M	var. *majus*	Ibworth, England	6,7
4/12/M	var. *majus*	England	6
53/2/M	var. *majus*	Scotland	6
30/3/M	var. *majus*	Ibworth, England	6,7
47/2/M	var. *majus*	Thirsk, England	6,7
AV9M	var. *majus*	Suffolk, England	7
SO54M	var. *majus*	Sotland	7
EST11	var. *majus*	Suffolk, England	7
NL195M	var. *majus*	Netherlands	7
NL209M	var. *majus*	Netherlands	7
MW3	var. *majus*	England	7
NL139M	var. *majus*	Netherlands	7
R17Db	var. *majus*	UK	7
SO33N	var. *nivale*	Scotland	7
SO51N	var. *nivale*	Scotland	7
Mn1/1	var. *nivale*	UK	7
SO47N	var. *nivale*	Scotland	7
-161/23	var. *nivale*	UK	7
M71	var. *nivale*	UK	7
44M	var. *majus*	Dumfries, Scotland	7
42N	var. *nivale*	East Lothian, Scotland	7

† = Isolated by Dr. J. Beck, Syngenta.

Appendix 3. PCR reaction ingredients for diagnostic PCR (25 µl reaction volume) and quantitative PCR (50µl reaction volume).

***Nucleotides* (1 or 2µl added)**
100µMof each deoxynucleotide tri phosphate (dCTP, dATP, dGTP, dTTP) (ABgene, UK)

***PCR buffer* (3.5 or 7 µl added)**

1.5mM	magnesium chloride (Sigma)
10mM	Tris-HCL (Sigma)
50mM	potassium chloride (Sigma)
100µg ml^{-1}	gelatine (Sigma)
0.05%	each of Tween 20 and Nonidet P-40 (Sigma)
5%	glycerol
40mg ml^{-1}	cresol red

***Primers* (0.05 or0.1 µl added)**
100nM each forward and reverse primer
***Polymerase enzyme* (0.1 or 0.2 µl added)**
0.5units/25µl super *Taq* polymerase (Kramel, UK)

Ultra purified water (Severn Biotech Ltd, UK)
15.3 µl diagnostic PCR
20.6 µl quantitative PCR

Appendix 4. Target species and genomic region, name, sequences and anneal temperatures for primers used in this study.

Species	Name/ Direction	Sequence	Anneal Temp °C	Genomic Region
N/A	EF1/F	(S)AT (Y)GA (R)AA GTT CGA GAA GG	52[a]	EF1α
N/A	EF2/F	(B)CT (Y)GA CAA GCT (S)AA	52[a]	EF1α
N/A	EF3/F	CGT GA(Y) TT(Y) ATC AA(R) AAC ATG	52[a]	EF1α
N/A	EF1/R	CC(R) ATC TTG TAG AC(R) TCC TGG	52[a]	EF1α
N/A	EF2/R	TGC AT(Y) TC(R) AC(R)GAC TTG A	52[a]	EF1α
M. nivale	EFMic/F	ATT GAG AAG TTC GAG AAG G	54[a]	EF1α
M. nivale	EFMic/R	CTT GTC GGT AGG ACG CTT G	54[a]	EF1α
M. nivale var. *majus*	EFMaj/F	CCC CTT CTC CCT ATC GC	54[b]	EF1α
M. nivale var. *nivale*	EFNiv/F	GTT CCC CTG TCT GAC TGT TGT	54[b]	EF1α
M. nivale	EFMniv/R	GTC TCG ATG GAG TCG ATG G	54[a]	EF1α
M. nivale	EFI*Sty*I/F	ATT GCG CCA TTC TCA TCA	54[a]	EF1α
Allium cepa	ONI/F	TGC TCT GCT GAT GTT GCC AG	58[c]	Alliinase gene
Allium cepa	ONI/R	TAC ATG GGG ATG GAG GTC TC	58[c]	Alliinase gene
Allium cepa	EFMaj/LF	TCC CTA TCG CTG CTG CTG CAA AGG	38[c]	Alliinase gene
Allium cepa	EFMNiv/LR	GAG TCG ATG GAG GTC GCG CAT GGT	38[c]	Alliinase gene
Allium cepa	EFNiv/LF	TGA CTG TTG TTG CTG CTG CAA AGG	38[c]	Alliinase gene
M. nivale	JBM/F	GGT GCT GTC TCT CGG GAC	60a	rDNA
M. nivale	JBM/R	TCC TCC GCT TAT TGA TAT GC	60a	rDNA
M. nivale var. *nivale*	NivITS/F	CTG TCT CTC GGG ACG GTG	54– 62[a]	rDNA
M. nivale var. *majus*	MajITS/F	GCT GTC TCT CGG GAC GGT A	54– 62[a]	rDNA
M. nivale	NGMic/R	GGT TTATGG CTG TTG CCA GTC	54– 62[a]	rDNA
Fusarium spp.	JBF/F	GTT TTT AGT GGA ACT TCT GAG T	60a	rDNA
Fusarium spp.	JBF/R	AAG TTG GGG TTT AAC GGC	60a	rDNA
Allium cepa	JBM/FL	CTC TCG GGA CGT TGC TCA TGC CCC	38[c]	rDNA
Allium cepa	JBM/RL	ATT GAT ATG CTC TCG GGA AGT GCC	38[c]	rDNA
Allium cepa	JBF/FL	ACT TCT GAG TAG GAA ATG CAG CGG	38[c]	rDNA
Allium cepa	JBF/RL	GTT TAA CGG CTG AGG TCG CGC ATG	38[c]	rDNA
M. nivale var. *nivale*	Y13NF	ACC AGC CGA TTT GTG GTT ATG	62[d]	Unknown
M. nivale var. *nivale*	Y13NR	GGT CAC GAG GCA GAG TTC G	62[d]	Unknown
M. nivale var. *majus*	Y13MF	CTT GAG GCG GAA GAT CGC	62[d]	Unknown
M. nivale var. *majus*	Y13MR	ATC CCT TTT CCG GGG TTG	62[d]	Unknown

N/A = Target species not applicable for degenerate primers
Letters in parenthesis indicate degenerate regions of primer sequences; (B = G/C/T, M = AC, R = A/G, S = GC, Y = C/T).
Underlined bases indicate bases in linker primers which are part of original primer sequences.
[(a-d)] Program type used (see appendix 5)

Appendix 5. PCR cycling conditions for the four PCR programs used in conjunction with primers described in Appendix 4.

Program A

Step number	Temp. (°C)	Time (min and s)
1	95	1 15
2	95	- 15
3	Anneal temp for primer (Appendix 4)	- 15
4	72	- 45
Repeat from step two 35 times		
5	72	4 15

Program B

Step number	Temp. (°C)	Time (min and s)
1	95	1 15
2	95	- 30
3	56	- 20
4	72	- 45
Repeat from step two five times		
5	95	- 30
6	55	- 20
7	72	- 45
Repeat from step five five times		
8	95	1 15
9	95	- 30
10	54	- 20
11	72	- 45
Repeat from step 9 25 times		
12	72	4 15

Program C

Step number	Temp. (°C)	Time (min and s)
1	95	1 30
2	95	- 30
3	38	- 20
4	72	- 40
Repeat from step two 20 times		
5	95	- 20
6	50	- 20
7	72	1 20
Repeat from step five 10 times		

Program D

Step number	Temp. (°C)	Time (min and s)
1	95	1 30
2	95	- 30
3	66	- 20
4	72	- 45
Repeat from step two five times		
5	95	- 30
6	64	- 20
7	72	- 45
Repeat from step five five times		
9	95	- 30
10	62	- 20
11	72	- 45
Repeat from step nine 25 times		
12	72	5 0

Appendix 6. Isolates used in Chapter 3 to examine specificity of EF-1α gene based *M. nivale* var. *majus* and var. *nivale* primers and results of comparisons between EF-1α based primers and primers described by Nicholson *et al.* (1996).

Code	Origin	Primers used/ sub-species	
		Nicholson *et al.*, 1996	EF-1α based primers
46M	United Kingdom	*majus*	*majus*
AV9M*	Suffolk, England	*majus*	*majus*
EST11	Suffolk, England	*majus*	*majus*
47/2/M	Thirsk, England	*majus*	*majus*
24/3/M	Ibworth, England	*majus*	*majus*
18/2/M	Bardney, England	*majus*	*majus*
1/1/M	Fyfield, England	*majus*	*majus*
30/3/M	Ibworth, England	*majus*	*majus*
SO54	Dumfries, Scotland	*majus*	*majus*
44M	Dumfries, Scotland	*majus*	*majus*
S048	East Lothian, Scotland	*majus*	*majus*
2/2/M	East Lothian, Scotland	*majus*	*majus*
S053	Midlothian, Scotland	*majus*	*majus*
NL007/1	Winschoten, Netherlands	*majus*	*majus*
NL015/1	Bedum, Netherlands	*majus*	*majus*
NL039/1	Beetgemermolen, Netherlands	*majus*	*majus*
NL110/2*	Heerlen, Netherlands	*majus*	*majus*
NL122/2	Goeree-Overflakkee, Netherlands	*majus*	*majus*
NL130/1	Sommelsdijk, Netherlands	*majus*	*majus*
NL136/1	Stad a.h. Haringvliet, Netherlands	*majus*	*majus*
NL160/2	Hoekschewaar, Netherlands	*majus*	*majus*
NL199/2	Zevenhuizen, Netherlands	*majus*	*majus*
F060/1	Somme, France	*majus*	*majus*
F070/2	Northern France	*majus*	*majus*
NG053/1*	Bad Segerberg, Northern Germany	*majus*	*majus*
SWG052/1	Lampertheim, Souh West Germany	*majus*	*majus*
S008/2	Switzerland	*majus*	*majus*
S010/2*	Switzerland	*majus*	*majus*
I035/1	Ireland	*majus*	*majus*
B001/1*	Middelbeke, Belgium	*majus*	*majus*
B009/1	Middelbeke, Belgium	*majus*	*majus*
NZ033/1	Marton, New Zealand	*majus*	*majus*
NZ00E/1	Wakanui, New Zealand	*majus*	*majus*
NZ00F/1	Darfield, New Zealand	*majus*	*majus*
NZ013/1	Methven, New Zealand	*majus*	*majus*
NZ011	Eiffelton, New Zealand	*majus*	*majus*
NZ3/JN	Monawatu, New Zealand	*majus*	*majus*
NZ024	Dunearn, New Zealand	*majus*	*majus*
NZ25/GVR	Monawatu, New Zealand	*majus*	*majus*
JP236880	Japan	*majus*	*majus*
NRRL 13303	USA	*majus*	*majus*
NRRL 13935	Washington, USA	*majus*	*majus*
NRRL 13934	Washington, USA	*majus*	*majus*
NRRL A-25906	Washington, USA	*majus*	*majus*

* Isolate used for sequencing EF-1α gene.

Appendix 6 (continued).

Code	Origin	Primers used/ result	
		Nicholson *et al.*, 1996	EF-1α based primers
139/2/N	Dernford, England	*nivale*	*nivale*
Nr4/N	Shropshire, England	*nivale*	*nivale*
117/1/N	Wye, England	*nivale*	*nivale*
4N	Cambridgeshire, England	*nivale*	*nivale*
36/1/N	Thirsk, England	*nivale*	*nivale*
74/1/N	North Yorkshire, England	*nivale*	*nivale*
18/1/N	Bardney, England	*nivale*	*nivale*
SO04N	Scotland	*nivale*	*nivale*
15N	Shropshire, England	*nivale*	*nivale*
59/1/N	Ibworth, England	*nivale*	*nivale*
68/2/N	Ibworth, England	*nivale*	*nivale*
129/1/N	Taunton, England	*nivale*	*nivale*
42N	East Lothian, Scotland	*nivale*	*nivale*
94/2/N	Shropshire, England	*nivale*	*nivale*
97/1/N	Thirsk, England	*nivale*	*nivale*
SO20	Fife, Scotland	*nivale*	*nivale*
SO48	East Lothian	*nivale*	*nivale*
SO28	Angus, Scotland	*nivale*	*nivale*
44/1	Dumfries, Scotland	*nivale*	*nivale*
SO53	East Lothian	*nivale*	*nivale*
2/1	East Lothian	*nivale*	*nivale*
B008/1*	Middelbeke, Belgium	*nivale*	*nivale*
NL005/1	Midwolda, Netherlands	*nivale*	*nivale*
NL090	Zandeweer, Netherlands	*nivale*	*nivale*
NL087	Zandeweer, Netherlands	*nivale*	*nivale*
NL102/9	Heerlen, Netherlands	*nivale*	*nivale*
NL122/1*	Goeree-Overflakkee	*nivale*	*nivale*
SWG047	Landshut, South East Germany	*nivale*	*nivale*
NG053	Bad Segeberg, Northern Germany	*nivale*	*nivale*
NG048/2*	Bad Segeberg, Northern Germany	*nivale*	*nivale*
F059	Oise, France	*nivale*	*nivale*
F056	Marne, France	*nivale*	*nivale*
F057	Somme, France	*nivale*	*nivale*
F060/4	Somme, France	*nivale*	*nivale*
JP101046	Japan	*nivale*	*nivale*
NRRL 3289	Winnipeg, Canada	D.N.A.	*nivale*
NZ013/5	Greendak, New Zealand	*nivale*	*nivale*
NZ006	Chertsey, New Zealand	*nivale*	*nivale*

* Isolate used for sequencing EF-1α gene.
D.N.A. = Did Not Amplify

Appendix 7. Location, variety, % *M. nivale* infection, var. *majus* and var. *nivale* DNA for seed lots used from 1997 harvest.

Lot No.	Location	Variety	% *M. nivale*	var. *majus* DNA (pg ng^{-1} total DNA)	var. *nivale* DNA (pg ng^{-1} total DNA)
1	Northumberland	Riband	6.5	ND	0.33
2	Perthshire	Riband	9	1.61	0.30
3	Inverness-shire	Riband	32.5	17.42	5.72
4	Yorkshire	Unknown	56.5	1.77	11.49
5	Worcester	Hunter	1.5	0.20	0.23
6	Suffolk	Consort	13.5	1.14	0.38
7	Suffolk	Unknown	21	ND	2.84
8	Berwickshire	Riband	28	5.26	2.34
9	Clackmannan	Riband	8.5	0.69	ND
10	Fife	Consort	22.5	3.28	1.74
11	Fife	Riband	69	41.87	3.59
12	Yorkshire	Unknown	54.5	4.36	10.40
13	Yorkshire	Rialto	88	60.63	37.50
14	Suffolk	Unknown	11.0	ND	0.982
15	Northumberland	Riband	87	31.32	3.44
16	Fife	Riband	29.0	2.98	0.25
17	Shropshire	Consort	5	0.13	0.83
18	Essex	Cadenza	1	ND	ND
19	Angus	Riband	60.5	14.02	1.94
20	Worcester	Riband	21.0	4.77	3.31
21	Angus	Riband	89.5	189.55	24.77
22	Worcester	Hunter	2	0.07	0.63
23	Worcester	Hereward	49.5	2.43	1.63
24	Angus	Riband	33.0	22.84	4.99
25	Perthshire	Riband	75.5	34.00	5.92
26	Worcester	Consort	5	0.11	ND
27	Worcester	Avans	3.5	ND	ND
28	Worcester	Chablis	11.0	0.61	ND
29	Warwickshire	Consort	36.0	0.92	0.60
30	Worcester	Cadenza	6.5	0.30	0.29
31	Worcester	Chablis	13.5	ND	ND
32	Aberdeenshire	Riband	18.0	1.44	0.40
33	Yorkshire	Unknown	31.0	1.00	4.78
34	Bannffshire	Riband	8.5	1.341	0.226
35	Berwickshire	Consort	36.5	3.655	1.45
36	Middlothian	Riband	15.5	11.63	5.53
37	Ross-shire	Consort	14.0	2.11	0.26
38	East Lothian	Charger	82.5	8.83	5.21
39	East Lothian	Riband	78.0	20.77	12.99
40	Warwickshire	Riband	30.0	1.19	5.42
41	Warwickshire	Reaper	0.5	ND	0.35
42	Warwickshire	Reaper	64	1.65	8.47
43	West Lothian	Riband	15.0	14.93	1.14
44	Middlothian	Madrigal	57.5	10.07	6.77
45	Dumfriesshire	Norman	45.0	33.53	2.97
46	Suffolk	Unknown	15.5	1.17	2.54
47	Fife	Riband	40.0	12.98	2.60

ND = Not Detected

Appendix 8. Location, variety, % *M. nivale* infection, var. *majus* and var. *nivale* DNA for seed lots used from 1998 harvest.

Lot No.	Location	Variety	% *M. nivale*	var. *majus* DNA (pg ng^{-1} total DNA)	var. *nivale* DNA (pg ng^{-1} total DNA)
1	Melrose	Riband	0.5	46.97	34.70
2	East Lothian	Riband	78.5	23.63	9.04
3	Berwick	Consort	55.5	ND	0.08
4	Didcot	Soissons	0.5	1.56	3.93
5	Hull	Unknown	3.5	0.46	1.25
6	Suffolk	Unknown	4.5	0.25	4.14
7	Suffolk	Unknown	6	3.23	3.61
8	Hull	Unknown	9.5	32.00	24.19
9	Suffolk	Unknown	68.5	4.02	5.16
10	Hull	Unknown	9.5	35.11	20.47
11	Suffolk	Unknown	60.5	0.68	0.14
12	Suffolk	Unknown	8.5	2.79	9.26
13	Worcester	Hussar	11	0.34	2.63
14	Suffolk	Unknown	7	0.81	0.40
15	Suffolk	Unknown	17.5	3.89	1.27
16	Suffolk	Unknown	7	0.20	0.06
17	Cambridge	Soissons	6	10.71	27.47
18	Suffolk	Unknown	54.5	1.58	4.07
19	Suffolk	Unknown	10	2.21	1.95
20	Suffolk	Unknown	5.5	1.77	2.30
21	Suffolk	Unknown	5.5	3.63	2.13
22	Hull	Unknown	20.5	8.12	8.48
23	Hull	Unknown	62.5	1.61	0.07
24	East Lothian	Consort	3	5.38	1.78
25	East Lothian	Rialto	4.5	0.90	2.28
26	Cambridge	Malacca	3	2.14	2.25
27	Worcester	Hussar	6	6.36	1.24
28	Berwick	Riband	14.5	0.40	3.69
29	Hull	Riband	4.5	2.48	5.25
30	Hull	Charger	9	0.36	0.81
31	Suffolk	Consort	6	1.65	1.69
32	Hull	Unknown	5	2.22	1.33
33	Hull	Unknown	9.5	2.76	2.00
34	Worcester	Hereward	8	0.61	0.26
35	Warickshire	Unknown	2	2.66	6.08
36	Hull	Unknown	25	2.85	11.99
37	Hull	Unknown	25	7.98	3.01
38	Berwick	Consort	46.5	9.89	4.51
39	Melrose	Riband	43.5	0.74	1.40
40	Worcester	Consort	9	1.02	1.41
41	Melrose	Riband	9.5	2.40	3.43
42	Fife	Consort	16	27.04	11.95
43	Dumfries	Riband	75.5	24.09	5.14
44	Dumfries	Consort	76.5	8.07	5.61
45	East Lothian	Abbot	34	4.07	2.22
46	Kelso	Kelso	21.5	8.50	2.52
47	Fife	Fife	47	46.97	34.70

ND = Not Detected

Appendix 9. Location, variety, % *M. nivale* infection, var. *majus* and var. *nivale* DNA for Scottish seed lots used from 1999 harvest.

Lot No.	Location	Variety	% *M. nivale*	var. *majus* DNA (pg ng^{-1} total DNA)	var. *nivale* DNA (pg ng^{-1} total DNA)
1	St. Boswells	Riband	9.0	ND	20.59
2	Kelso	Riband	32.5	9.60	19.86
3	Ross-shire	Riband	13.0	3.97	8.80
4	St. Boswells	Riband	1.0	ND	0.65
5	Perth	Riband	8.5	0.77	11.62
6	Kinross	Riband	1.0	ND	0.56
7	Kinross	Riband	3.5	ND	0.31
8	Kinross	Riband	18.5	2.84	8.17
9	Arbroath	Riband	1.0	0.66	0.46
10	Angus	Buchan	13.0	3.42	6.44
11	Falkirk	Riband	10.5	1.27	10.61
12	Falkirk	Riband	1.5	0.57	10.68
13	Fife	Riband	5.0	ND	1.48
14	Falkirk	Consort	17.0	3.21	9.56
15	Haddington	Riband	4.0	ND	1.52
16	Angus	Claire	15.5	4.03	4.68
17	Angus	Lynx	6.0	ND	0.41
18	Berwickshire	Riband	18.0	2.03	19.39
19	Blairgowrie	Savannah	19.5	3.51	13.49
20	Arbroath	Riband	3.5	1.06	0.80
21	Tranent	Unknown	20.5	0.91	7.63
22	Tranent	Riband	2.5	ND	1.99
23	Cupar	Riband	6.5	2.38	3.44
24	Fife	Riband	27.5	13.09	4.93
25	Angus	Riband	8.5	0.64	1.63
26	Fife	Unknown	4.0	1.05	1.25
27	Inverness	Malacca	17.0	15.09	6.67
28	Ross-shire	Buchan	51.0	6.84	55.21
29	Ross-shire	Lynx	4.0	ND	1.16
30	Ross-shire	Hunter	14.0	1.44	13.73
31	East Lothian	Riband	53.5	5.31	44.17
32	Haddington	Consort	10.5	0.68	5.86
33	Haddington	Rialto	27.0	5.74	24.77
34	Peterhead	Unknown	49.0	25.62	88.26
35	Tranent	Riband	34.0	6.59	20.23
36	Tranent	Riband	5.5	2.09	2.28
37	Perth	Madrigal	8.0	3.54	5.43
38	Perth	Riband	7.0	2.51	4.15
39	Ellon	Riband	8.5	2.24	4.03
40	Kelso	Consort	4.5	ND	0.59
41	Kelso	Riband	5.5	0.40	1.08
42	Cupar	Riband	4.5	0.79	0.56
43	Perth	Riband	2.5	ND	2.22
44	Haddington	Lynx	41.0	10.79	30.58

Appendix 9 (continued).

Lot No.	Location	Variety	% *M. nivale*	var. *majus* DNA (pg ng^{-1} total DNA)	var. *nivale* DNA (pg ng^{-1} total DNA)
45	Crieff	Riband	26.0	ND	31.34
46	Haddington	Consort	35.0	1.70	24.16
47	Clackmannan	Riband	10.5	6.78	ND
48	Clackmannan	Savannah	2	1.55	ND
49	Clackmannan	Savannah	1.5	1.33	1.21
50	Cupar	Consort	21.5	9.36	4.62
51	Cupar	Hunter	33	25.30	7.49
52	Fife	Consort	1.5	4.93	0.73
53	Fife	Riband	3	1.71	ND
54	Haddington	Claire	8.5	3.10	3.54
55	Fife	Riband	30.5	11.37	2.30
56	St. Andrews	Consort	2	1.76	ND
57	St. Andrews	Riband	2	3.08	ND
58	Haddington	Riband	27	20.52	1.24
59	Oldmeldrum	Riband	32	19.13	2.13
60	Haddington	Abbot	14	13.18	2.10
61	Angus	Riband	11	2.18	1.73
62	Fife	Riband	43.5	32.24	0.83
63	Fife	Riband	16.5	7.62	2.71
64	Haddington	Imp	6	4.15	0.92
65	Haddington	Chablis	6.5	2.38	1.00
66	Blairgowrie	Chablis	2.5	1.70	1.66
67	Haddington	Imp	19.5	5.27	0.63

Appendix 10. Percentage *M. nivale* infection, *M. nivale* var. *majus* and var. *nivale* DNA for English seed lots 1-40 from 1999 harvest. All samples originated from Herefordshire/Worcestershire, variety was not given.

Lot No.	% *M. nivale*	Sub-species/ amount of DNA (pg ng^{-1} total DNA)		Lot No.	% *M. nivale*	Sub-species/ amount of DNA (pg ng^{-1} total DNA)	
		var. *majus*	var. *nivale*			var. *majus*	var. *nivale*
1	1.5	0.09	ND	**21**	3.5	1.54	0.59
2	3	0.31	ND	**22**	3.5	3.86	0.74
3	3.5	3.02	0.98	**23**	5.5	4.63	7.33
4	6.5	7.49	2.80	**24**	7	2.17	ND
5	3	1.54	0.68	**25**	6.5	7.00	2.12
6	2.5	0.97	0.51	**26**	6	0.64	0.76
7	2.5	2.06	0.49	**27**	0.5	1.30	1.10
8	6	2.48	5.27	**28**	2.5	1.45	ND
9	7	2.31	3.40	**29**	0.5	1.37	1.00
10	0.5	0.20	0.65	**30**	2	0.85	0.39
11	2.5	1.69	1.30	**31**	0.5	1.31	1.68
12	1.5	0.12	ND	**32**	0.5	ND	1.06
13	1	0.61	1.24	**33**	5	2.65	2.97
14	6.5	2.80	0.50	**34**	2	1.23	0.75
15	1.5	0.34	ND	**35**	3.5	1.61	ND
16	5.5	0.85	0.50	**36**	6.5	4.10	2.57
17	2.5	0.44	0.28	**37**	1.5	0.99	0.43
18	3	1.15	0.45	**38**	6	4.52	1.40
19	4.5	1.06	0.26	**39**	0.5	1.05	0.47
20	6.5	7.94	1.92	**40**	2	2.07	0.56

Location, variety, % *M. nivale* infection, var. *majus* and var. *nivale* DNA for English seed lots 41-63 from 1999 harvest.

Lot No.	Origin	Variety	% *M. nivale*	var. *majus* DNA (pg ng^{-1} total DNA)	var. *nivale* DNA (pg ng^{-1} total DNA)
41	Market Rasen	Claire	6.5	4.31	0.89
42	Market Rasen	Hereward	7.5	2.95	0.82
43	Market Rasen	Equinox	1.0	1.54	1.01
44	Market Rasen	Malacca	4.0	ND	ND
45	Market Rasen	Consort	1.5	2.54	0.93
46	Suffolk	Unknown	2.0	1.41	1.53
47	Suffolk	Unknown	2.0	1.08	0.94
48	Market Rasen	Savannah	1.5	ND	ND
49	Suffolk	Unknown	3.5	5.02	ND
50	Suffolk	Unknown	3.0	1.42	ND
51	Newark	Equinox	12.5	2.31	2.18
52	Newark	Riband	1.5	0.81	0.31
53	Newark	Consort	0.5	1.44	0.61
54	Newark	Hereward	1.0	0.90	1.39
55	Newark	Rialto	1.5	0.67	ND
56	Chippenham	Savannah	1.5	1.18	0.48
57	Chippenham	Riband	4.5	0.92	0.93
58	Suffolk	Unknown	1.5	0.91	ND
59	Suffolk	Unknown	2.0	2.44	0.94
60	Suffolk	Unknown	1.0	2.40	1.15
61	Suffolk	Unknown	1.5	1.27	0.76
62	Warwickshire	Consort	13.5	7.03	10.53
63	Warwickshire	Abbot	2.0	1.12	1.14

ND = Not detected

Appendix 11. Mean, Maximum and Minimum daily temperatures over course of seedling blight experiment.

Days after sowing	Mean Temperature (°C)	Max Temperature (°C)	Minimum Temperature (°C)
1	7.5	16.8	2.6
2	11.5	17.1	8
3	10.6	19.5	8.5
4	11.0	14.8	9.3
5	8.9	14.8	5.1
6	10.8	18	8.5
7	10.5	19.2	7.5
8	8.5	15.7	5.4
9	7.2	9.8	3.9
10	8.2	13.7	5.7
11	6.8	13.4	3.4
12	6.5	14	4.6
13	6.7	12.6	3.6
14	6.4	13.4	3.4
15	5.7	12.8	2.6
16	8.2	13.1	6.9
17	9.1	13.4	7.5
18	7.4	9.8	5.9
19	7.3	11.2	5.9
20	6.8	11.5	3.1
21	9.0	14.2	6.9
22	7.6	13.1	5.1
23	6.1	12.8	3.4
24	3.8	9.8	1.1
25	4.5	8.8	0.8
26	6.8	9	4.9
27	3.8	9.6	1.1
28	5.2	10.1	2.3
29	9.0	11.7	6.7
30	4.5	9	1.1
31	2.3	8.8	-0.9
32	5.4	8.3	3.4
33	4.7	8	2.6
34	4.5	8.3	1.1
35	6.3	9.8	4.9
36	6.9	10.4	5.4
37	4.8	9.3	2.8
38	10.3	14.5	8.3
39	11.4	15.1	9.6
40	7.3	12.6	4.6
41	10.3	13.7	9
42	8.7	13.1	6.7
43	6.3	11.7	3.9
44	9.2	12	7.7
45	10.6	12.8	9
46	9.3	12.3	7.2
47	8.1	11.2	6.4

Mean Temperature = 7.5
Mean Maximum temperature = 12.4°C
Mean minimum temperature = 5.0°C

Overall Maximum = 19.5°C
Overall Minimum = -0.9°C

Appendix 12. Accumulated analysis of variance for quantitative PCR data from 1998 and 1999 field trials.

Quantification of *M. nivale* at GS12 in 1998 field trial

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ pcr	2	0.08641	0.04320	1.60	0.207
+ fungicid	2	9.56177	4.78088	176.91	<.001
+ seedlot	3	6.84554	2.28185	84.43	<.001
+ pcr.fungicid.seedlot	28	3.56514	0.12733	4.71	<.001
Residual	108	2.91871	0.02703		
Total	143	22.97756	0.16068		

Quantification of *Fusarium* spp. at GS12 in 1999 field trial

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ pcr	2	1.4127	0.7063	1.90	0.155
+ fungicid	2	114.8886	57.4443	154.20	<.001
+ seedlot	3	77.1235	25.7078	69.01	<.001
+ pcr.fungicid.seedlot	28	31.6569	1.1306	3.03	<.001
Residual	108	40.2327	0.3725		
Total	143	265.3143	1.8553		

Quantification of *M. nivale* at GS25 in 1998 field trial

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ pcr	2	0.03574	0.01787	0.62	0.540
+ fungicid	2	3.14522	1.57261	54.56	<.001
+ seedlot	3	0.93835	0.31278	10.85	<.001
+ pcr.fungicid.seedlot	28	0.82123	0.02933	1.02	0.453
Residual	108	3.11282	0.02882		
Total	143	8.05337	0.05632		

Quantification of *M. nivale* at GS12 1999 field trial

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ pcr	2	0.07687	0.03843	0.82	0.445
+ fungicid	2	38.88606	19.44303	412.67	<.001
+ seedlot	3	19.65900	6.55300	139.08	<.001
+ pcr.fungicid.seedlot	28	12.31904	0.43997	9.34	<.001
Residual	108	5.08843	0.04712		
Total	143	76.02940	0.53167		

Quantification of *M. nivale* var. *nivale* at GS12 in 1999 field trial

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ pcr2	1	0.1885	0.1885	0.70	0.408
+ fungicid	1	1.1745	1.1745	4.37	0.044
+ seedlot	2	1.6558	0.8279	3.08	0.058
+ pcr2.fungicid.seedlot	7	0.4607	0.0658	0.25	0.971
Residual	36	9.6643	0.2685		
Total	47	13.1438	0.2797		

Quantification of *M. nivale* var. *majus* at GS12 in 1999 field trial

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ pcr2	1	0.0723	0.0723	0.30	0.585
+ fungicid	1	10.5830	10.5830	44.52	<.001
+ seedlot	2	2.6662	1.3331	5.61	0.008
+ pcr2.fungicid.seedlot	7	1.9689	0.2813	1.18	0.337
Residual	36	8.5576	0.2377		
Total	47	23.8481	0.5074		

Quantification of *M. nivale* at GS25 in 1999 field trial

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ pcr	2	0.0967	0.0483	0.37	0.692
+ fungicid	2	7.0857	3.5428	27.12	<.001
+ seedlot	3	14.4224	4.8075	36.80	<.001
+ pcr.fungicid.seedlot	28	3.9829	0.1422	1.09	0.365
Residual	108	14.1086	0.1306		
Total	143	39.6962	0.2776		

Quantification of *M. nivale* var. *nivale* at GS25 in 1999 field trial

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ pcr2	1	0.0006	0.0006	0.00	0.953
+ fungicid	2	2.6586	1.3293	7.84	<.001
+ seedlot	3	6.3255	2.1085	12.44	<.001
+ pcr2.fungicid.seedlot	17	3.0863	0.1815	1.07	0.398
Residual	72	12.2008	0.1695		
Total	95	24.2718	0.2555		

Quantification of *M. nivale* var. *majus* at GS25 in 1999 field trial

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ pcr2	1	0.00000	0.00000	0.00	0.996
+ fungicid	2	3.61778	1.80889	27.19	<.001
+ seedlot	3	3.64028	1.21343	18.24	<.001
+ pcr2.fungicid.seedlot	17	0.68137	0.04008	0.60	0.879
Residual	72	4.78988	0.06653		
Total	95	12.72931	0.13399		

Quantification of *M. nivale* var. *nivale* at GS59 in 1999 field trial

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ pcr2	1	0.03508	0.03508	0.65	0.423
+ fungicid	2	4.21783	2.10891	38.97	<.001
+ seedlot	3	3.11878	1.03959	19.21	<.001
+ pcr2.fungicid.seedlot	17	2.07482	0.12205	2.26	0.009
Residual	72	3.89620	0.05411		
Total	95	13.34271	0.14045		

Quantification of *M. nivale* var. *majus* at GS59 in 1999 field trial

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ pcr2	1	0.00033	0.00033	0.01	0.930
+ fungicid	2	1.28593	0.64296	15.19	<.001
+ seedlot	3	0.91837	0.30612	7.23	<.001
+ pcr2.fungicid.seedlot	17	1.03106	0.06065	1.43	0.147
Residual	72	3.04806	0.04233		
Total	95	6.28375	0.06614		

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